REMARKS

In a non-final Office Action with a mailing date of May 23, 2006, the Examiner acknowledges that claims 3-9 and 16-21 were pending in the application, and that claims 3-9 and 16-21 are rejected. Applicants now respond to this Office Action and respectfully request that the Examiner, in consideration of the claim amendments and remarks included herein, place claims 3-9 and 16-21 in condition for allowance.

Claims 16-17 Rejected Under 35 U.S.C. § 112, Second Paragraph

Claims 16 and 17 stand rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention. Applicants have amended claims 16 and 17 so as to more clearly recite their relationship to the claims from which they depend.

Applicants' respectfully request that claims 16 and 17 be entered into the record and found in condition for allowance.

Claims 5, 8-9, and 18-21 Rejected Under 35 U.S.C. § 112, First Paragraph

Claims 5, 8-9 and 18-21 stand rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement. The Office Action cites to in re Wands and a listing of Wands factors used to gauge the enablement of the given disclosure.

1. The Office Action states, in part that, "Nature of the invention: The invention relates to genetically engineering the somatic stem cells with retroviral vectors and engrafting or *ex vivo* therapy with said engineered cells". Office Action, page 3.

2. The Office Action states, in part, that, "The specification does not present enabled evidences for broad claims on methods of engraftment of any type of cells (i.e. xenotransplantations, etc.) to other than non-human mammals". *Id.* at 4.

3. The Office Action states, in part, that the level of ordinary skill in the art is, "high requiring an advanced degree or training in the relevant field". *Id.*

4. The Office Action states, in part, that, "At about the effective filing date of the present application [the] art is unpredictable with regard to gene therapy and methods of *in vivo* gene transfers". *Id.* In support of this position the Office Action cites a review by Goncalves *et al.*, Bioessays, 2005, 27:506-517). *Id.*

5. The Office Action concludes that, "These claims are not enabled because one of skill in the art would not be able to rely upon the state of the art in order to successfully predict a priori the *in vivo* all the effects of the retroviral vectors or the retroviral-transduction of transgene or its fragments in a subject". *Id.* at 5.

Applicants respectfully disagree with this assessment of the enabling disclosure and the state of the art with respect to *ex vivo* treatment of human disease at the time that the instant application was filed. As acknowledged in the Office Action, the level of proficiency of those of skill in the art in this area is extremely high. Accordingly, the skill possessed by those skilled in the art is also high; therefore, the disclosure of the application at the time of filing was sufficient to enable those skilled in the art to practice the invention.

The Office Action characterizes the invention as being drawn to "engraftment or *ex vivo* gene therapy" *Id.* at 4. Later the Office Action states that "Gene therapy or *in vivo* gene transfers are still considered to be highly experimental area of research..." *Id.*

at 4. The review article cited by the Office Action is largely drawn to assessments of *in vivo* gene therapy. As illustrated by specific examples cited below in the review article by Goncalves *et al.*, drawing on a large body of results gleaned from *in vivo* gene therapy trials draws an unduly pessimistic view of the use of *ex vivo* therapy to treat human disease.

The Applicants submit for the Examiner's consideration three scientific papers, all published in peer-reviewed scientific journals. These papers are included in Exhibit A (attached). These papers are as follows: (A) "Sustained Correction of X-Linked Severe Combined Immunodeficiency by *Ex Vivo* Gene Therapy", by Salima Hacein-Bey-Abina, *et al.*, The New England Journal of Medicine, volume 346, April 18, 2002, p. 1185-1193; (B) "T Lymphocyte-Directed Gene Therapy for ADA SCID: Initial Trial Results After 4 Years" by R. Michael Blaese *et al.*, Science, vol. 270, October 20, 1995, p. 475-480; (C) "Successful *ex vivo* gene therapy directed to liver in a patient with familial hypercholesterolaemia" by Mariann Grossman *et al.*, Nature Genetics, volume 6, April 1994, p. 335-341.

Salima Hacein-Bey-Abina *et al.* is directed to the use of *ex vivo* retrovirus transduction to treat x-linked severe combined immunodeficiency due to changes in the human γ (γc) chain. This paper reports on the successful treatment of four boys who suffered from this genetic condition by use of *ex vivo* retroviral gene therapy. *Id.* at 1190. The effectiveness of using *ex vivo* retroviral gene therapy to treat disease is summarized in the paper. See *Id.* at 1191. Specific portions of the Salima Hacein-Bey-Abina paper include descriptions of materials and methods which clearly illustrate that use of

retroviral transductions of human cells to treat human diseases was enabled on or about the time that the instant application was filed. *See Also Id.* at 1180.

Sill another example of the successful use of *ex vivo* gene therapy to treat genetic defects in humans can be found in the paper to Blaese, R.M. *et al.* *See e. g.* Blaese R.M. *et al.*, abstract and pg. 475. This paper states that, "This trial of retroviral-mediated gene transfer shows that the survival of reinfused transduced peripheral blood T cells is prolonged *in vivo*; the erroneous assumption that T cells would not have such long-term survival was often cited as a potential problem with the treatment strategy." *Id.* at 478, col. 2, paragraph 1. The Examiner is also invited to review the rest of Blaese for additional evidence that the use of *ex vivo* retroviral transduction to treat human disease was enabled in the art on or before the time when the instant application was filed.

Applicants also wish to bring to the attention of the Examiner the Grossman, M. *et al.* paper, published in April, 1984. This paper also reports on the successful use of *ex vivo* transduction of human hepatocytes to successfully treat a genetic liver disorder in a human patient. *See e. g.,* Grossman, M., *et al.*, the Abstract on page 335. Grossman states that, "In this strategy, stable reconstitution of hepatic gene expression can be achieved by transplanting hepatocytes transduced *ex vivo* with retroviruses," and also that, "However, there should be no immunological barriers associated with *ex vivo* gene therapy other than the problem of an immune response to the therapeutic gene product, a potential concern that is generic to all forms of gene therapy for deficiency states." *Id.* at 339. Grossman also states that, "The outcome of the first clinical experience supports the safety and feasibility of *ex vivo* gene therapy directed to liver. Molecular and metabolic data suggests that the genetically-modified hepatocytes have engrafted stably in this

patient and continue to express the recombinant gene (after at least eighteen months)." *Id.* at 340. It is also of note that in these references cited, no host rejection of *ex vivo* transformed cells re-transplanted into the patient was observed.

As evidenced by the above-mentioned peer reviewed articles, *ex vivo* gene therapy was successfully used to treat human diseases or conditions caused by genetic defects in various cell types at about the time when the application was filed. Some embodiments of the instant application are directed towards materials and methods for increasing the efficiency and safety of retroviral transductions for subsequent engraftment into humans. These references clearly show that *ex vivo* treatment of human disease was sufficiently advanced at the time that the instant application was filed to enable the practice the invention. Accordingly, the Applicants respectfully request that all rejections for non-enablement be removed and the claims be placed in condition for allowance.

Claims 3-5, 8-9 and 16-21 Rejected Under 35 U.S.C. § 102 (a)

Claims 3-5, 8-9 and 16-21 were also rejected under 35 U.S.C. § 102 (a) as being anticipated by Kiem *et al.*, (1995, Curr. Opin, Oncol 7:107-14). Kiem *et al.* teaches that transduction is performed by cocultivation. Kiem *et al.* states that "improved *in vitro* transduction was achieved by combining cocultivation and retrovirus exposure in a long-term marrow culture system for 4 days". *See* Kiem *et al.*, pg. 108, col. 2, lns. 26-28. In contrast, claims 3 and 5 of the instant application as currently amended, recite in part, "wherein said infection is performed without the cocultivation in the presence of retroviral producer cells". Accordingly, in view of the amendments made to claims 3 and 5, the rejection of the claims as anticipated by Kiem *et al.* are mute and should be removed.

Additionally, there is nothing disclosed in Kiem *et al.* that teaches or suggests leaving polycationic molecules out of in *ex vivo* retrovirus cell transduction media or materials. Accordingly, Kiem *et al.* does not anticipate nor make obvious the claims of the instant application and the Applicants respectfully request that all pending claims including amended claims be placed in condition of allowance.

Claims 3-5, 8-9 and 16-21 Rejected under 35 U.S.C. § 103(a)

The claims also stand rejected under as being obvious over the following references cited by the Examiner: Moritz *et al.* (1994, The Journal of Clinical Investigation 93:1451-1457) and Nolta *et al.* (1996, Proc. Natl., Acad. Sci. USA 93:2414-2419) in view of Papp *et al.* (1987 Biochim, Biophys. Acta 925:241-247). A fair reading of Papp *et al.* shows that it only discloses that polybrene inhibits the binding of fibronectin to gelatin. Papp *et al.* does not teach or suggest that polybrene has any effect on the binding of retroviruses to cells; furthermore it provides no motivation to test the effect of leaving polybrene or other polycations out of retroviral transduction media. Accordingly, Papp *et al.* in combination with Moritz *et al.* and/or Nolta *et al.*, does not make obvious the Applicants' claimed invention and the Applicants respectfully request that this rejection be removed and that all pending claims be placed in condition for allowance.

Claims 3 and 4 Rejected Under the Judicially Created Doctrine of Nonstatutory Type

Finally, claims 3 and 4 are rejected on the grounds of judicially created doctrine of obviousness-type double patenting. The Office Action rejects claims 3 and 4 under this doctrine as being unpatentable over claims 2 and 19 of U.S. Patent No. 6,670,177 B2, and claims 20-22 of U.S. Patent No. 5,686,278. The Office Action also notes that these rejections could be overcome by timely filing of a terminal disclaimer. Applicants would entertain filing a terminal disclaimer over these two U.S. patents should all other rejections and objections to the pending claims be removed.

Conclusion

In view of the aforementioned claim amendments and remarks, Applicants respectfully request that all pending claims 3-9 and 16-21 in the application be placed in condition for allowance. Action to that end is respectfully requested.

If there are any issues in this case which could be more efficiently addressed telephonically, the Examiner is invited to please contact the undersigned telephonically to discuss these matters.

Respectfully submitted,

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EXHIBITS (Attachments)

(A) "Sustained Correction of X-Linked Severe Combined Immunodeficiency by *Ex Vivo* Gene Therapy", by Salima Hacein-Bey-Abina, *et al.*, The New England Journal of Medicine, volume 346, April 18, 2002, p. 1185-1193

(B) "T Lymphocyte-Directed Gene Therapy for ADA SCID: Initial Trial Results After 4 Years" by R. Michael Blaese *et al.*, Science, vol. 270, October 20, 1995, p. 475-480

(C) "Successful *ex vivo* gene therapy directed to liver in a patient with familial hypercholesterolaemia" by Mariann Grossman *et al.*, Nature Genetics, volume 6, April 1994, p. 335-341.

Exhibit A

The New England Journal of Medicine

VOLUME 346 APRIL 18, 2002 NUMBER 16

SUSTAINED CORRECTION OF X-LINKED SEVERE COMBINED IMMUNODEFICIENCY BY EX VIVO GENE THERAPY

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ABSTRACT

Background X-linked severe combined immunodeficiency due to a mutation in the gene encoding the common γ (γc) chain is a lethal condition that can be cured by allogeneic stem-cell transplantation. We investigated whether infusion of autologous hematopoietic stem cells that had been transduced in vitro with the *γc* gene can restore the immune system in patients with severe combined immunodeficiency.

Methods CD34+ bone marrow cells from five boys with X-linked severe combined immunodeficiency were transduced ex vivo with the use of a defective retroviral vector. Integration and expression of the *γc* transgene and development of lymphocyte subgroups and their functions were sequentially analyzed over a period of up to 2.5 years after gene transfer.

Results No adverse effects resulted from the procedure. Transduced T cells and natural killer cells appeared in the blood of four of the five patients within four months. The numbers and phenotypes of T cells, the repertoire of T-cell receptors, and the in vitro proliferative responses of T cells to several antigens after immunization were nearly normal up to two years after treatment. Thymopoiesis was documented by the presence of naive T cells and T-cell antigen-receptor episomes and the development of a normal-sized thymus gland. The frequency of transduced B cells was low, but serum immunoglobulin levels and antibody production after immunization were sufficient to avoid the need for intravenous immunoglobulin. Correction of the immunodeficiency eradicated established infections and allowed patients to have a normal life.

Conclusions Ex vivo gene therapy with *γc* can safely correct the immune deficiency of patients with X-linked severe combined immunodeficiency. (N Engl J Med 2002;346:1185-93.)

DEFICIENCY of the common γ (γc) chain, an X-linked disorder, causes the most frequent form of severe combined immunodeficiency disease.[1,2] The γc chain is an essential component of five cytokine receptors, all of which are necessary for the development of T cells and natural killer cells. Without the γc chain, there is a complete absence of mature T and natural killer cells, whereas B cells are usually present in normal or increased numbers. Severe combined immunodeficiency is fatal during the first year of life because of severe, recurrent infections, unless transplantation of hematopoietic stem cells restores T-cell function.[3,4] The survival rate after transplantation of HLA-identical hematopoietic stem cells is more than 90 percent, whereas with haploidentical stem cells it is 70 to 78 percent.[3,4] In most patients, deficient B-cell function persists after transplantation and requires lifelong immune-globulin–replacement therapy.[3,5] Some patients also have persistent deficiencies of T-cell function after stem-cell transplantation.[4,6] Assessment of an al-

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Other authors were E. Graham Davies, M.D., Great Ormond Street Hospital for Children, National Health Service Trust, London; Wietse Kuis, M.D., Ph.D., Department of Immunology and Hematology, Wilhelmina Kinderziekenhuis Lundlaan, Utrecht, the Netherlands; and Lilly Leiva, Ph.D., Department of Pediatrics, Louisiana State University Health Science Center, New Orleans.

ternative therapy based on the ex vivo transfer of the γc gene into autologous hematopoietic precursor cells was therefore warranted. In a preliminary report, we showed that this approach corrected the T-cell deficiency in two patients with X-linked severe combined immunodeficiency who were followed for 10 months after gene transfer.[7] We now report the effectiveness of the procedure in five patients with a follow-up of up to 30 months.

METHODS

Patients

Five consecutive patients without HLA-identical donors were enrolled in the trial between March 1999 and February 2000. The main characteristics of these boys at the time of diagnosis are shown in Table 1. The diagnosis of X-linked severe combined immunodeficiency was based on peripheral-blood lymphocyte counts and confirmed by γc mutation analysis. The protocol was approved by the French Drug Agency and the local ethics committee, and written informed consent was obtained from the parents, who were told that an alternative treatment (bone marrow transplantation) was available. All of the patients were kept in sterile isolation and received nonabsorbable antibiotics and intravenous immune globulin. Additional information about the five patients is available as Supplementary Appendix 1 with the full text of this article at http://www.nejm.org.

Retrovirus-Mediated Transduction

The vector containing the γc chain was derived from a defective Moloney murine leukemia virus and has been previously described.[7] With the patients under general anesthesia, 30 to 150 ml of bone marrow was obtained, and CD34+ cells in the marrow were selected for, as described below. These cells were stimulated to grow in X-vivo 10 medium (BioWhittaker, Walkersville, Md.) containing 4 percent fetal-calf serum (StemCell Technologies, Vancouver, B.C., Canada), 300 ng of stem-cell factor per milliliter (Amgen, Thousand Oaks, Calif.), 300 ng of Flt-3 ligand per milliliter (Immunex, Seattle), 60 ng of interleukin-3 per milliliter (Novartis, Rueil-Malmaison, France), and 100 ng of polyethylene glycol–conjugated megakaryocyte growth and differentiation factor per milliliter (Amgen). The cells were then transduced with a supernatant of the cultured γc-containing vector in the presence of the preceding cytokines and 4 ng of protamine sulfate per milliliter (Choay Sanofi, Gentilly, France). The procedure was carried out in sterile bags (Nexell Therapeutics, Irvine, Calif.) that were coated with 50 ng of human recombinant fibronectin per milliliter (Takara Shuzo, Shiga, Japan). The supernatant was replaced every 24 hours during the three-day transduction period. The number of cultured cells was increased by a factor of five to eight, and 14 million to 38 million CD34+ cells per kilogram of body weight were infused into the patients without preparative conditioning (Table 1).

Analysis of Immune Reconstitution

Immunofluorescence analysis, assays for proliferation of peripheral-blood mononuclear cells, analysis of the T-cell–receptor repertoire, and studies of natural-killer-cell cytotoxicity were performed as previously described.[7-9] The presence of serum antibodies against polioviruses, tetanus and diphtheria toxoids, *Haemophilus influenzae,* and *Streptococcus pneumoniae* was determined by enzyme-linked immunosorbent assays. Levels of isohemagglutinins were measured by a hemagglutination assay. Antibody levels were determined one to three months after three immunizations had been administered. The interval between the last intravenous infusion of immune globulin and the determination of antibody levels was at least three months.

TABLE 1. CHARACTERISTICS OF THE PATIENTS.

PATIENT NO.	AGE AT TREATMENT	CLINICAL STATUS BEFORE TREATMENT	ENGRAFTMENT OF MATERNAL T CELLS	MUTATION	γc EXPRESSION BEFORE TREATMENT	INFUSED CELLS		CLINICAL STATUS AFTER TREATMENT	FOLLOW-UP
						CD34+	CD34γc+		
	mo		cells/mm³			cells/kg			yr
1	11	*Pneumocystis carinii* pneumonitis Protracted diarrhea Failure to thrive	0	Arg 289→stop	Yes	15 million	7 million–14 million	Well Normal growth	2.5
2	8	*Pneumocystis carinii* pneumonitis Protracted diarrhea Graft-versus-host disease–like lesions Failure to thrive	<10	Deletion of exon 6	No	16 million	5 million	Well Normal growth	2.3
3	10	Disseminated bacille Calmette–Guérin infection Adenovirus and respiratory syncytial virus infections in the lungs Protracted diarrhea Failure to thrive	0	Deletion of exon 4	No	14 million	5 million	Improving*	0.7
4	1	Well Free of infection	0	Tyr 219→stop	No	27 million	14 million	Well Normal growth	1.8
5	3	Graft-versus-host disease–like lesions	2000	Gln 285→Ala	No	38 million	20 million	Well Normal growth	1.6

*Eight months after gene therapy, Patient 3 underwent allogeneic stem-cell transplantation.

Leukocyte Subgroups and Purification of CD34+ Cells

Peripheral-blood samples were separated into mononuclear cells and granulocytes by centrifugation and sorted by flow cytometry (FACS Vantage, Becton Dickinson Immunocytometry Systems, San Jose, Calif.). Isolation of CD34+ progenitor cells was performed by an immunomagnetic procedure (Miltenyi Biotec, Bergisch Gladbach, Germany). Two successive immunomagnetic procedures increased the purity of the CD34+ population to 99 percent.

Quantification of Transgene Integration

Genomic DNA was extracted from peripheral-blood mononuclear cells and amplified with use of quantitative polymerase chain reaction (PCR). Amplification, data acquisition, and analysis were performed with the use of a sequence detector (ABI PRISM 7700, Perkin Elmer, Norwalk, Conn.). Two sets of primers and probes were used in each PCR reaction. For the quantification of integrated transgene sequences, the primers positioned in the long terminal repeat and probe were as previously described.[10] The standard curve used as a reference for quantification of the viral copy number was based on serial dilutions of a plasmid ranging from 40 to 4 million copies. This plasmid contained two copies of the long terminal repeat and one of the human albumin sequence (Genethon III Laboratory, Evry, France).

To define the detection limit and linear range of duplex PCR, we used a standard curve consisting of a log-scale dilution of cells from an Epstein–Barr virus (EBV)–transformed B-cell line derived from a patient with X-linked severe combined immunodeficiency and containing approximately two copies of γc provirus per cell with uninfected cells from the same EBV-transformed B cell line. The lower limit of sensitivity of the method was 0.01 percent of γc-positive cells.

Quantification of T-Cell Antigen-Receptor Episomes

Analysis of T-cell antigen-receptor episomes in peripheral-blood mononuclear cells was performed by real-time quantitative PCR by means of the 5′ nuclease assay (TaqMan) with an ABI PRISM 7700 system (Perkin Elmer).[11,12] PCR conditions as well as primers and probe sequences are available on request.

Presence of Integrated Provirus after Long-Term Culture of CD34+ Cells

Purified CD34+ cells were cultured for six weeks on irradiated MS-5 stromal feeder layers in a limiting-dilution assay (10,000 to 150 cells per well) as described previously.[13] After six weeks, the cells were assayed for colony-forming units. Subsequently, for each dilution, all colony-forming units obtained on day 14 from the same dish were pooled. DNA was analyzed by PCR to determine the percentage of γc-positive dishes.

RESULTS

Clinical Outcome

After infusion of CD34+ cells that had been transduced in vitro with the γc gene, four of the five patients (Patients 1, 2, 4, and 5) had a clear-cut clinical improvement (Table 1). Pulmonary infections in Patient 1 and Patient 2 cleared and did not recur, and graft-versus-host–like skin lesions, a feature of severe combined immunodeficiency, disappeared in Patient 2 and Patient 5 within the first 50 days after gene therapy. Patient 1 and Patient 2 left the sterile environment on day 90, and Patient 4 and Patient 5 left on day 45. In Patient 1 and Patient 2, protracted diarrhea resolved, and parenteral nutrition was discontinued four months and three months after gene therapy, respectively. None of these four patients have subsequently had severe infections. Intravenous immune globulin was discontinued three to four months after gene therapy. Growth and psychomotor development have been normal to date. Patients 1, 2, 4, and 5 are now living at home in normal environmental conditions.

Patient 3, in whom reconstitution of T cells failed, underwent splenectomy four months after gene therapy for persistent splenomegaly caused by a disseminated bacille Calmette–Guérin infection. A rescue stem-cell transplantation from an unrelated donor matched at HLA-A, B, DR, and DQ loci but mismatched at one HLA-C locus was performed after eight months, according to the protocol. At the last follow-up visit, partial T-cell immunity had been restored in this patient.

T-Cell Development

In Patients 1, 2, and 4, the number of T cells increased progressively and reached normal values for age three to four months after gene therapy; they were within the normal range at the last follow-up visit (Fig. 1). In Patient 5, the initially high number of maternal T cells (Table 1) disappeared within three months after treatment, while autologous T cells appeared.

Quantitative analysis of provirus integration indicated that 100 percent of the T cells from Patients 1, 2, 4, and 5 contained the transgene (Fig. 2). On Southern blotting, there were one to three provirus integration sites per cell (data not shown). All T cells in Patients 2, 4, and 5 expressed cell-surface recep-

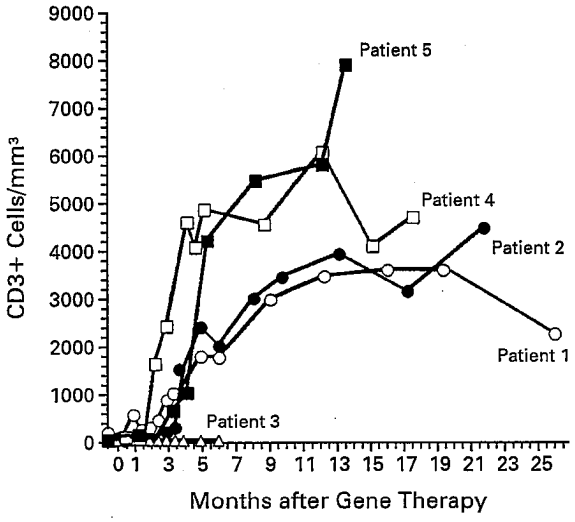

Figure 1. Absolute Numbers of CD3+ Cells after Gene Transfer in Patients 1 through 5.

tors with the γc chain. In all four patients, there was a normal distribution of T cells with α/β or γ/δ receptors, and the numbers of CD4+ and CD8+ T cells were similar to those in age-matched controls (data not shown). Conversely, no T cells were detected in the blood of Patient 3 up to six months after treatment (Fig. 1).

Analysis of naive (CD45RA+) and memory (CD45RO+) subgroups within CD4+ and CD8+ populations showed that most T cells had the phenotype of naive CD45RA+ T cells (Fig. 3A). We also assessed whether T cells were being synthesized by measuring the level of T-cell antigen-receptor episomes. Intrathymic rearrangements of genes encoding T-cell antigen receptors cause the formation of extrachromosomal DNA episomes, which mark T cells that have recently emigrated from the thymus to the periphery. As shown in Figure 3B, T-cell antigen-receptor episomes in Patients 1, 2, and 4 were first detected between day 60 and day 90, reached values found in age-matched controls, and remained stable for up to two years after gene transfer. Thirteen months after treatment, Patient 5 had 5500 CD45RA+ CD4+ T cells per cubic millimeter and 21,000 T-cell antigen-receptor episomes per 100,000 peripheral-blood mononuclear cells, respectively. These data correlated well with the development of a normal-sized thymus, as evaluated by ultrasonography (in Patients 1, 2, 4, and 5) and by magnetic resonance imaging in Patient 5 (respective size at one year or more, 23 by 15 by 11.5 mm, 21 by 13 by 10 mm, 27 by 34 by 13 mm, and 19 by 15 by 7 mm) (Fig. 3C).

Expression of 17 Vβ families of T-cell receptors in

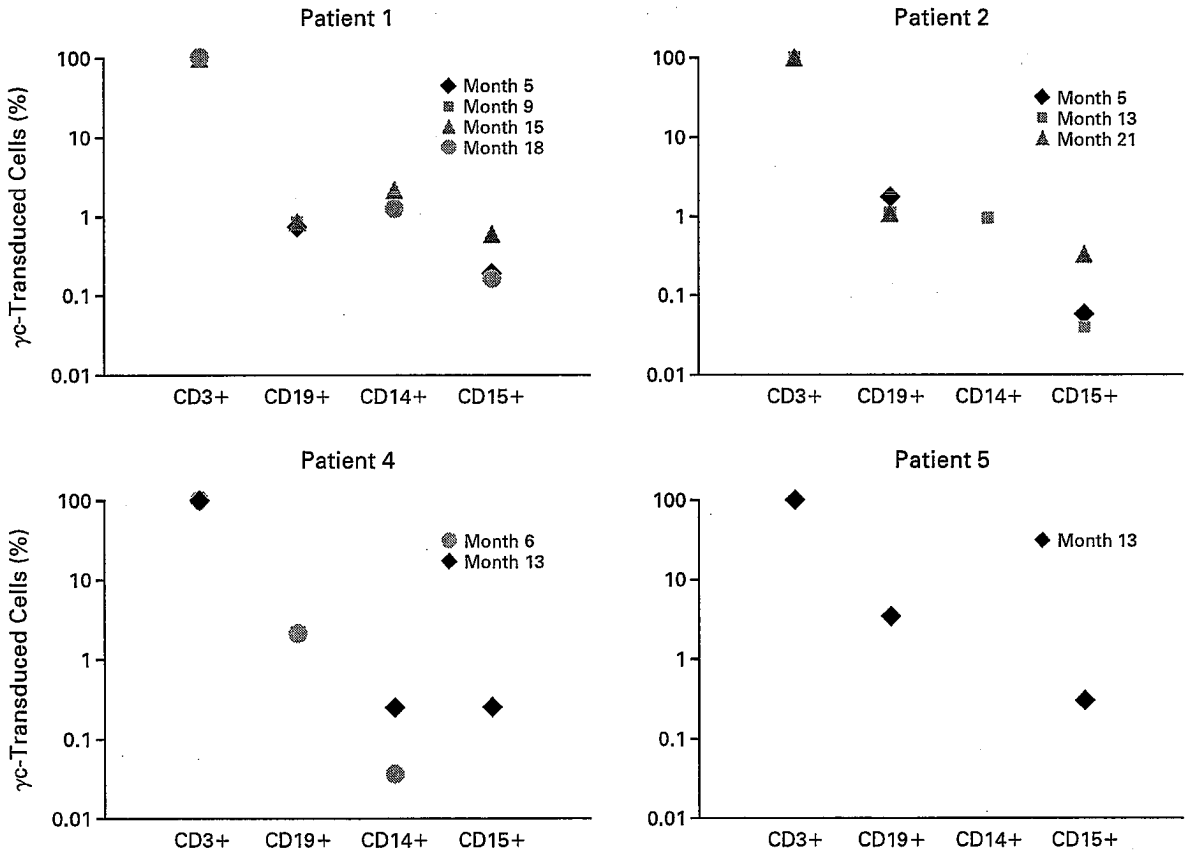

Figure 2. Frequency of Sorted T Cells (CD3+), B Cells (CD19+), Monocytes (CD14+), and Granulocytes (CD15+) Containing the Common γ (γc) Chain after Gene Therapy in Patients 1, 2, 4, and 5.

Real-time quantitative polymerase-chain-reaction analysis of DNA was used to determine the frequency of vector-containing cells, as described in the Methods section.

Patients 1, 2, 4, and 5 was similar to that in age-matched controls, and in these patients CD4+ and CD8+ T-cell populations remained stable. In all patients, a gaussian distribution of the lengths of complementarity-determining region 3 for 22 tested Vβ families of T-cell receptors was observed (see Supplementary Appendix 1).

Capacity for T-Cell Proliferation

At the last follow-up visit, T cells from Patients 1, 2, 4, and 5 exhibited normal proliferative responses to in vitro stimulation with phytohemagglutinin and anti-CD3 antibody (see Supplementary Appendix 1). Antigen-specific proliferative T-cell responses were also observed after immunization of those four patients with tetanus toxoid and polioviruses (see Supplementary Appendix 1). The addition of interleukin-2 to T cells from Patients 4 and 5 enhanced in vitro proliferative responses to tetanus toxoid. T cells from Patient 1, who was immunized with bacille Calmette–Guérin at two months of age, also had a proliferative response to tuberculin (purified protein derivative).

Development of Natural Killer Cells

Natural killer cells became detectable 15 to 45 days after gene therapy in Patients 2, 4, and 5 and 150 days after gene therapy in Patient 1 (Fig. 4). In Patients 2 and 4, and to a lesser magnitude in Patient 5, the levels of natural killer cells peaked two to four months after gene therapy and then gradually decreased. In Patient 3, natural killer cells were also detected in the blood beginning on day 45. These cells expressed γc as detected by immunofluorescence analysis (see Supplementary Appendix 1) and exhibited cytotoxic activity against K562 target cells (data not shown).

Serum Immunoglobulins and Antibody Production

Serum IgG, IgA, and IgM levels at 25, 21, and 13 months in Patients 1, 2, and 5, respectively, were

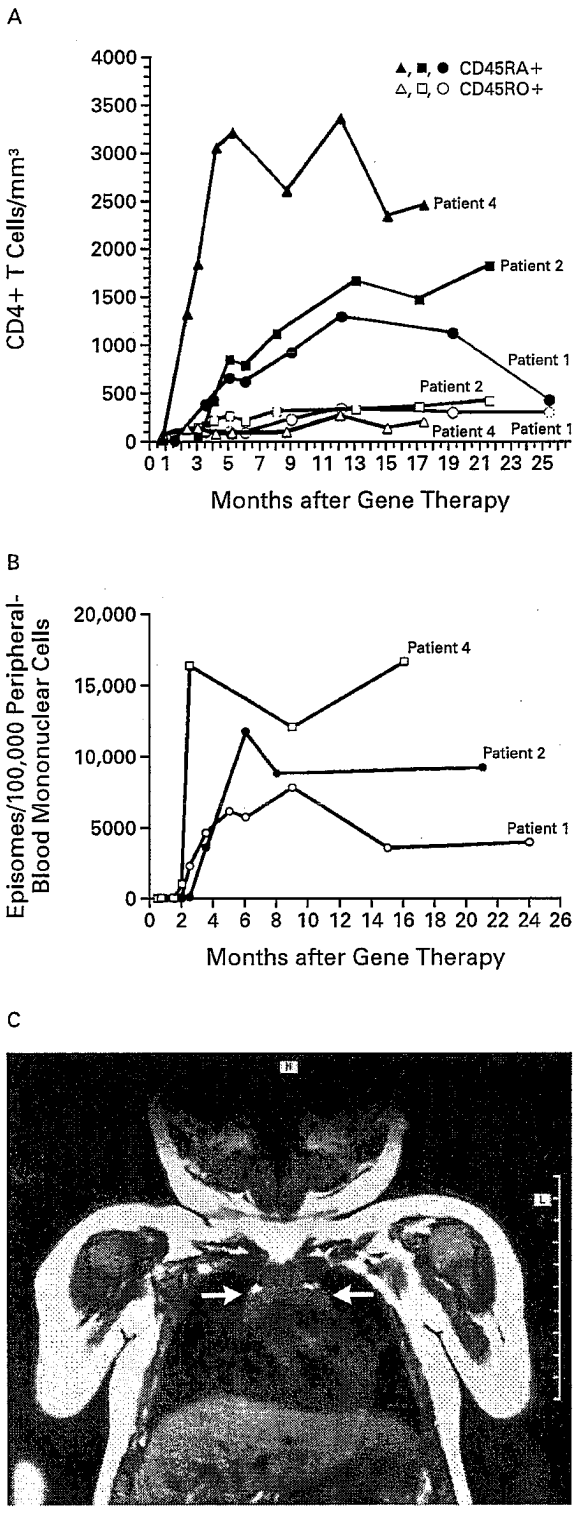

Figure 3. Numbers of Naive (CD45RA+) and Memory (CD45RO+) T Cells (Panel A) and Numbers of T-Cell Antigen-Receptor Episomes (Panel B) after Gene Therapy in Patients 1, 2, and 4 and Magnetic Resonance Image of a Coronal Section of the Thymus in Patient 5 Five Months after Gene Therapy (Panel C).

In Panel A, phenotypic quantification of naive and memory CD4+ T cells was performed with the use of double staining with fluorochrome-conjugated antibodies against CD4 and CD45RA or CD45RO. In Panel B, numbers of T-cell antigen-receptor episomes in peripheral-blood mononuclear cells were evaluated at different times. The normal range of T-cell antigen-receptor episomes for age-matched controls is 2500 to 20,000 per 100,000 peripheral-blood mononuclear cells. Arrows in Panel C show a normal-sized thymus after reconstitution of T cells.

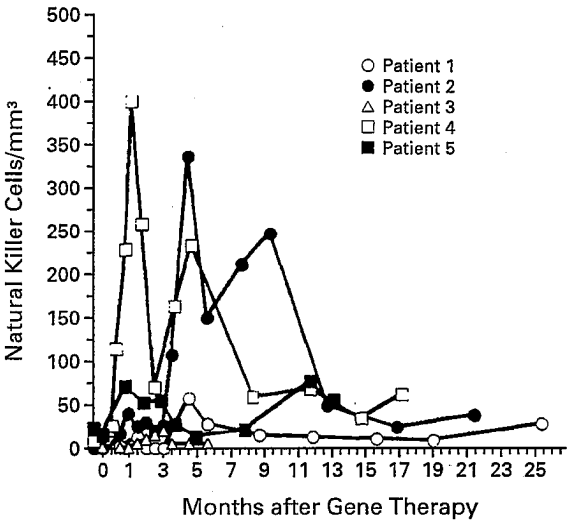

Figure 4. Absolute Numbers of CD56+ and CD16+ Cells per Cubic Millimeter of Whole Blood after Gene Therapy in Patients 1 through 5.

within the age-related normal range (Fig. 5). Low IgG and IgA levels persisted in Patient 4 (Fig. 5). Antibodies against tetanus toxoid, diphtheria toxoid, and poliovirus antigens were first found one month after the third immunization (Table 2) and persisted for more than six months in Patients 1, 2, and 4. Antibodies against *S. pneumoniae* in Patient 2 and *H. influenzae* in Patient 1 and Patient 2 were also detected. In contrast, immunization of Patient 5 failed to elicit an antibody response. Isohemagglutinins were consistently detected in the serum of Patients 1, 2, and 4 one year or more after gene therapy (Table 2). In three patients, the percentage of CD27+ and CD19+ B cells was similar to that of age-matched controls (see Supplementary Appendix 1).

Integration and Expression of γc Provirus

In Patients 1, 2, 4, and 5, all CD3+ T cells carried the *γc* transgene, as compared with 1 to 5 percent of B cells, 0.05 to 2 percent of monocytes, and 0.05 to 0.5 percent of granulocytes (Fig. 2). The frequency of *γc*-containing T cells, B cells, monocytes, and granulocytes was stable during the study period (Fig. 2). In Patients 2, 4, and 5, the presence of the *γc* gene coincided with the expression of γc chains (see Supplementary Appendix 1). In bone marrow samples obtained from Patient 2 and Patient 4 21 and 13 months, respectively, after gene transfer, 1 to 5 percent of colony-forming units derived from cultured CD34+ cells contained the transgene (frequency of long-term-culture initiating cells, 1:1000 in Patient 2 and 1:500 in Patient 4) (data not shown).

Patient 3

Reconstitution of T cells failed to occur in Patient 3 (Fig. 1), despite the presence of *γc*-positive cells, as detected by PCR and immunofluorescence analysis of peripheral-blood mononuclear cells from day 30 up to four months after gene transfer. After splenectomy, a strong *γc* signal was detected among sorted CD19+ and CD16+ cells by nonquantitative PCR analysis. There were no CD3+ T cells in the spleen, and provirus (i.e., vector) was not detected in a bone marrow sample obtained at the time of splenectomy.

DISCUSSION

We found that four of five patients with X-linked severe combined immunodeficiency due to a deficiency of the γc chain who were treated with autologous CD34+ cells from bone marrow that had been transduced ex vivo with the *γc* gene showed evidence of a functional immune system and sustained clinical benefit. These results extend a preliminary report of two patients treated in this way.[7] The gene-therapy protocol we used is safe, and no evidence of the emergence of a replication-competent retrovirus has been detected.

The evidence that virtually all T cells and natural killer cells but fewer B cells and myeloid cells were transduced suggests that *γc* expression gives progenitors of T cells and natural killer cells a selective growth advantage. Since transduced monocytes, granulocytes, and colonies derived from long-term cultures of transduced CD34+ cells were consistently detected one to two years after gene transfer, it is likely that long-lived immature progenitor cells were targeted by the vector. Moreover, the persistence of T-cell antigen-receptor episomes,[11,12] naive T cells, and the development of a normal-sized thymus indicate ongoing formation of T cells and thymopoiesis, which most likely originated from transduced CD34+ progenitors. These findings suggest that both committed myeloid and lymphoid progenitor cells were transduced (implying that these cells persist in the bone marrow for at least one to two years) or that uncommitted pluripotent progenitor cells were transduced by the *γc*-containing vector. Evaluation of provirus integration sites in myeloid and lymphoid cells[14,15] should help clarify this issue.

In our four successfully treated patients, the pattern of restoration of T cells differed from that observed after transplantation of haploidentical hematopoietic stem cells in patients with severe combined immunodeficiency.[3,4] After the latter, T cells usually begin to appear within four to six months, and the

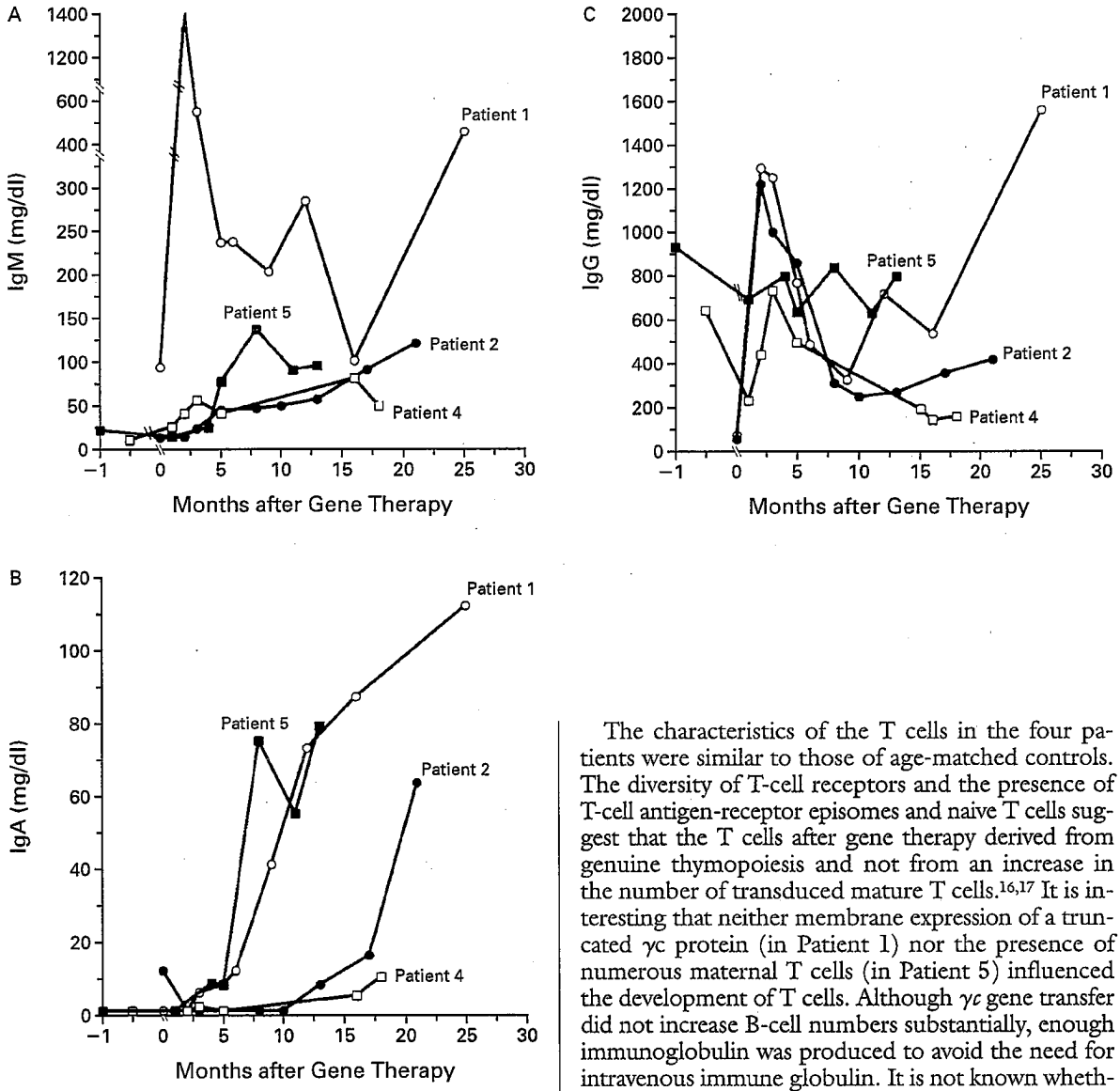

Figure 5. Serum Levels of IgM (Panel A), IgA (Panel B), and IgG (Panel C) after Gene Therapy in Patients 1, 2, 4, and 5.

In Patient 1, the peak level of monoclonal IgM occurred two months after gene therapy.

number of T cells in peripheral blood rarely exceeds 2000 per cubic millimeter.[3,4] In contrast, after gene therapy, T cells appeared within two to four months, at levels of 2000 to 8000 per cubic millimeter. The absence of graft-versus-host disease and the ex vivo activation of CD34+ cells with cytokines could have contributed to the rapid reconstitution.

The characteristics of the T cells in the four patients were similar to those of age-matched controls. The diversity of T-cell receptors and the presence of T-cell antigen-receptor episomes and naive T cells suggest that the T cells after gene therapy derived from genuine thymopoiesis and not from an increase in the number of transduced mature T cells.[16,17] It is interesting that neither membrane expression of a truncated γc protein (in Patient 1) nor the presence of numerous maternal T cells (in Patient 5) influenced the development of T cells. Although γc gene transfer did not increase B-cell numbers substantially, enough immunoglobulin was produced to avoid the need for intravenous immune globulin. It is not known whether the few transduced B cells account for the production of antibodies in these patients or whether nontransduced B cells are also involved.[18] Since there were more detectable memory B cells (CD27+ and CD19+) than transduced B cells, it is possible that γc-negative B cells retain some function.

In conclusion, our study demonstrates that the infusion of autologous γc-transduced cells, despite the low efficiency of the transduction process, can repair the immune system in patients with X-linked severe combined immunodeficiency. Although the repair is incomplete, it is sufficient to provide protective immunity. Despite an obvious requirement for long-term assessment and further analysis in a larger cohort of patients, these results suggest that a similar approach

TABLE 2. PEAK ANTIBODY RESPONSES AFTER IMMUNIZATION.*

ANTIBODY ASSAY	PATIENT 1	PATIENT 2	PATIENT 4	PATIENT 5	CONTROLS
Diphtheria toxoid (IU/ml)	3	93	22	<0.1	>0.10
Tetanus toxoid (IU/ml)	3	63	89	<0.1	>0.10
Poliovirus titer					
First	1:640	1:640	1:20	0	>1:40
Second	1:320	1:640	1:80	1:20	>1:40
Third	1:160	1:160	1:40	0	>1:0
Anti-A antibody titer	1:64	1:32	1:8	1:4	>1:8
Anti-B antibody titer	1:32	—	—	—	—
Haemophilus influenzae (%)†	26	16	ND	ND	>10
Streptococcus pneumoniae (μg/ml)	ND	8	ND	ND	>0.3

*Patients were immunized three times with diphtheria toxoid, tetanus toxoid, and poliovirus between month 4 and month 6; they were immunized with *Streptococcus pneumoniae* and *Haemophilus influenzae* one year after gene therapy. Serum antibodies were measured in serum samples drawn every three months thereafter. ND denotes not done.

†A positive value is more than 10 percent.

could be used for other forms of severe combined immunodeficiency.[19-24]

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We are indebted to the families of the patients for their continuous support of the study; to the medical and nursing staff of the Unité d'Immunologie et d'Hématologie Pédiatriques, Hôpital des Enfants Malades, for patient care; to Jean-Laurent Casanova, Geneviève de Saint Basile, and Anne Durandy for their contribution to the study; to L. Coulombel for helpful advice; to F. Gross, P. Nussbaum, C. Harre, C. Jacques, and F. Selz for technical help; to S. Yoshimura and I. Kato (Takara Shugo, Shiga, Japan) for providing the CD-296 fibronectin fragment; to B. Bussière, C. Caillot, and J. Caraux (Amgen, France) for providing stem-cell factor and megakaryocyte growth and development factor; and to P. Johnson and D. Louis for editorial assistance.

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CORRECTION

Gene Therapy for Severe Combined Immunodeficiency Disease

To the Editor: Correction of X-linked severe combined immunodeficiency by infusion of autologous CD34+ stem cells transduced with retrovirus containing common γ chain, reported by Hacein-Bey-Abina et al. (April 18 issue),[1] is a milestone in medicine. We used a different therapy with a similarly good outcome.

X-linked severe combined immunodeficiency was diagnosed in two patients after the initiation of mechanical ventilation for pulmonary failure caused by infections. Immediately after the diagnosis had been made, haploidentical CD34+ peripheral progenitor cells mobilized with granulocyte colony-stimulating factor were isolated to a purity of more than 99 percent.[2] These cells were infused with no preparative regimen and no prophylaxis against graft-versus-host disease. Both patients showed signs of T-cell reconstitution beginning three weeks after the CD34+ infusion and were weaned from the ventilator. They are in excellent health, without graft-versus-host disease, 34 and 68 months after transplantation. Patient 1 does not need replacement immune globulin. Patient 2 received a "booster" infusion of CD34+ stem cells from the original donor one year later to improve B-cell function and now receives immune globulin every three months.

Our experience indicates that purified haploidentical CD34+ progenitor cells reconstitute the T-cell compartment and can correct the B-cell defect. Given the possibility of long-term risks[3,4] and the availability of effective alternatives, we think that broader application of gene therapy for the treatment of patients with severe combined immunodeficiency or strategies for the correction of persistent B-cell deficiency after successful allogeneic transplantation[5] are premature and warrant longer follow-up.

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The authors reply:

To the Editor: Handgretinger et al. describe two cases of successful haploidentical hematopoietic stem-cell transplantation for X-linked severe combined immunodeficiency. It is indeed known that partially compatible hematopoietic stem-cell transplantation can provide T-cell reconstitution in 70 to 80 percent of cases.[1,2] Nevertheless, haploidentical hematopoietic stem-cell transplantation has a number of pitfalls. Despite low numbers of T cells in the graft, graft-versus-host disease does develop in some cases (5 to 10 percent). T-cell repopulation after haploidentical hematopoietic stem-cell transplantation is slow.[1,2] A period of more than three months is usually required before T cells can be detected. More important, Patel et al. have reported that after the performance of haploidentical hematopoietic stem-cell transplantation without myeloablation, T-cell immunity declines over time.[3] Finally, correction of B-lymphocyte immunity is infrequent in patients with X-linked severe combined immunodeficiency who undergo haploidentical hematopoietic stem-cell transplantation in the absence of myeloablation.[1,4] In contrast, so far all patients who have received gene therapy, with a follow-up of more than one year, in whom T-cell immunity has developed do not require intravenous immune globulin therapy. These observations justify further assessment of gene therapy as an alternative to hematopoietic stem-cell transplantation.

The potential risk of gene therapy must not be underestimated and must be balanced against the risk of alternative therapy. The concern of Handgretinger et al. is not entirely appropriate, since helper virus and the expression of a membrane receptor, which accounted for reported toxic effects, are irrelevant to our trial. In our opinion, gene therapy can be considered an option worth exploring for patients with severe combined immunodeficiency.

We would also like to note that on page 1185 of our article, author Lily Leiva's name was misspelled, and the affiliation for Dr. Leiva and author Ricardo Sorensen should have included both Louisiana State University Health Sciences Center and Children's Hospital, New Orleans.

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becco s minimum essential medium (DMEM) in the presence of polybrene (8 μg/ml) as described (*18*). Cells were washed twice in phosphate-buffered saline (PBS), resuspended in fresh medium, and cultured for 3 to 4 days. Transduced cells were tested for the presence of helper virus and cryopreserved until use.

36. BM mononuclear cells were obtained as a Ficoll fraction and grown for 2 to 3 days in complete DMEM at a density of 6×10^5 to 8×10^5 cells/cm^2 (*35*). T cell depletion and progenitor cell enrichment were obtained as described (*3, 35*). Gene transfer was carried out by multiple infection cycles with cell-free, helper virus–tested viral supernatants in the presence of polybrene (8 μg/ml) (*35*). BM cells were maintained in a long-term culture system over adherent layers without addition of exogenous growth factors, and infected during the first 3 days of culture. Transduced cells were tested for the presence of helper virus and cryopreserved until use. At that time, the transduced cells were washed, resuspended in normal saline containing 4% human albumin, and reinfused into the patient.
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39. The relative frequencies of transduced BM progenitor cells were obtained by comparing the frequency of CFU-G, CFU-GM, BFU-E, and CFU-GEMM cells in the absence and presence of increasing doses of G418 (0.7 1.0, 1.5 mg/ml) as described (*37*). In selected experiments, individual G418-resistant colonies were collected for analysis of vector transduction and expression.
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49. We are indebted to L. Ruggieri and A. Wack for performing some of the ex vivo and in vitro analyses of gene transfer frequency; to the nurses and clinical staff of the Clinica Pediatrica, School of Medicine, University of Brescia, for skilled and dedicated care; to A. Arrighini and A. Crescenzo for clinical assistance in the extended care of the two patients; to A. Plebani for dosing specific antibody production; to M. Hershfield, P. Dellabona, and A. Ballabio for helpful discussions; and to Enzon, Inc., and Ophan Europe for providing PEG-ADA before commercial distribution. Supported by grants from Telethon, the Italian National Research Council, and the Italian Ministry of Health (IV-VII AIDS Projects).

26 May 1995; accepted 27 September 1995

T Lymphocyte–Directed Gene Therapy for ADA$^-$ SCID: Initial Trial Results After 4 Years

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In 1990, a clinical trial was started using retroviral-mediated transfer of the adenosine deaminase (ADA) gene into the T cells of two children with severe combined immunodeficiency (ADA$^-$ SCID). The number of blood T cells normalized as did many cellular and humoral immune responses. Gene treatment ended after 2 years, but integrated vector and ADA gene expression in T cells persisted. Although many components remain to be perfected, it is concluded here that gene therapy can be a safe and effective addition to treatment for some patients with this severe immunodeficiency disease.

The possibility of using gene transfer as a therapy for human disease has great appeal. The decision to enter clinical trials awaited the development of safe and efficient techniques of gene transfer and improved understanding of the basic pathology and biology underlying likely candidate diseases and target cells. The advent of useful retroviral vectors that permitted relatively high efficiency gene transfer and stable integration was a critical advance (*1*, *2*), as was the demonstration that this procedure of gene transfer could be effectively and safely used in humans (*3*).

Severe combined immunodeficiency secondary to a genetic defect in the purine catabolic enzyme adenosine deaminase [ADA$^-$ SCID] is characterized by defective T and B cell function and recurrent infections, often involving opportunistic pathogens. Large amounts of deoxyadenosine, an ADA substrate, are present in these patients; deoxyadenosine is preferentially converted to the toxic compound deoxyadenosine triphosphate in T cells, disabling the immune system (*4*).

Because this disease is curable by allogeneic bone marrow transplantation given without pretransplantation cytoreductive conditioning, it was initially assumed that gene therapy should be directed at the bone marrow stem cell. However, initial attempts to use stem cell gene transfer in primates resulted in only low-level, transient gene expression, insufficient for clinical use. The observation that the only donor cells detected in some patients "cured" by allogeneic bone marrow transplantation was their T cells—the others remaining ADA-deficient (*5*)—raised the possibility that T cell–directed gene therapy also might be a useful treatment.

The introduction of enzyme replacement with ADA-containing erythrocytes (*6*) or with bovine ADA conjugated with polyethylene glycol (PEG-ADA) (*7*) has made this approach feasible. PEG-ADA has provided noncurative, life-saving treatment for ADA$^-$ SCID patients; with this treatment, most patients have experienced weight gain and decreased opportunistic infections. Full immune reconstitution has been less regularly achieved with enzyme therapy. T cell function as measured by in vitro mitogen responses improved in most patients, but fewer patients recovered consistent immune responses to specific antigens [for instance, as measured by normal delayed-type hypersensitivity (DTH) skin test reactivity] (*8–10*). Nearly all PEG-ADA–treated patients showed increased peripheral T cell counts, which provided a source of T cells for gene correction not available without enzyme therapy. Furthermore, enzyme treatment could be continued during the gene therapy trial so that the ethical dilemma of withholding or stopping a life-saving therapy to test an unknown treatment could be avoided.

The adenosine deaminase complementary DNA (cDNA) (*11*) is 1.5 kb and fits within a retroviral vector. With the use of an ADA-containing retroviral vector, ADA-deficient T cell lines were transduced to express normal amounts of ADA; this rendered them normally resistant to intoxication and growth inhibition when challenged with deoxyadenosine (*12, 13*). Next, studies in mice, rabbits, and nonhuman primates using T cells modified with retroviral vectors showed normal cell survival and function after their reintroduction into recipient animals (*14*). Finally, Bordignon and colleagues (*15*) showed that ADA gene–corrected T cells acquired a survival advantage compared with uncorrected ADA-deficient cells when transplanted into immunodeficient, but ADA-

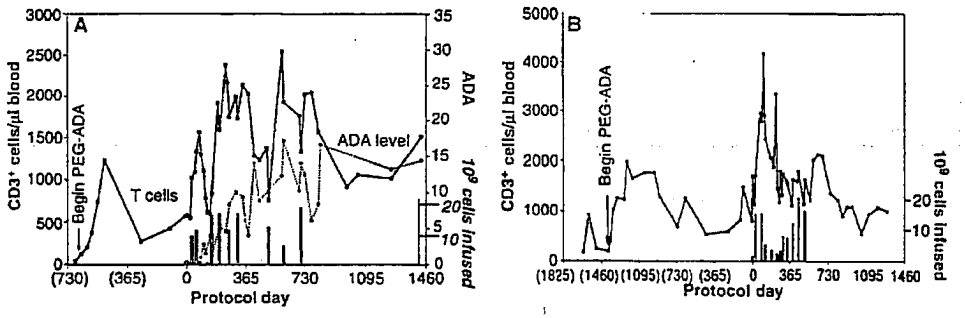

Fig. 1. Peripheral blood T cell counts since the time the diagnosis of ADA deficiency was made, dates of treatments, and the total number of cells infused for each patient. ADA level is measured in nanomoles of adenosine deaminated per minute per 10^8 cells. Vertical bars indicate the dates of cell infusion, and their height represents the total number of nonselected cells infused at each treatment. The T cell numbers represent total CD3-bearing T cells determined by standard flow cytometric analysis. (A) Patient 1 began gene therapy on 14 September 1990 (protocol day 0) and received a total of 11 infusions. Cellular ADA enzyme level is indicated by the dashed line. ADA activity was determined as described (*13*, *25*). Values shown are the mean of duplicate samples and represent EHNA-sensitive ADA enzyme activity. (B) Patient 2 began gene therapy on 31 January 1991 (protocol day 0) and received of a total of 12 infusions.

normal BNX recipient mice.

The clinical protocol used here has been described elsewhere (*16*). Patients with documented ADA$^-$ SCID were eligible if they did not have a human lymphocyte antigen–matched sibling as a potential donor for marrow transplantation and if they had been treated with PEG-ADA for at least 9 months without full immune reconstitution. T cells were obtained from their blood by apheresis, induced to proliferate in culture, transduced with the ADA retroviral vector LASN, culture-expanded, and then reinfused into the patient after 9 to 12 days (*17*). No selection procedure was used to enrich for gene-transduced cells.

The clinical histories and ADA gene mutations of each patient have been reported (*18*, *19*). Patient 1 presented with infection at 2 days of age and had recurrent infections and very poor growth until 26 months of age, when the diagnosis of ADA deficiency was established and she was started on PEG-ADA [30 U per kilogram of body weight per week (30 U/kg/week)]. Treatment with PEG-ADA enzyme for approximately 2 years had resulted in significant, but incomplete, benefit. With PEG-ADA she gained weight, had fewer infections, and transiently developed a normal peripheral blood T cell count (Fig. 1A), and her T cells had acquired the ability to respond to mitogens in vitro. However, significant immune deficiency persisted, including recurrence of her T lymphopenia (Fig. 1A), DTH skin test anergy (Table 1), depressed in vitro immune reactivity to specific antigens such as tetanus toxoid, failure to generate normal cytotoxic T cells to viral antigens or allogeneic cells, defective immunoglobulin production and absent or weak antibody responses to several vaccine antigens, and borderline isohemagglutinin titers (Table 1). At 4 years of age, she was enrolled in this trial.

The course of disease in patient 2 (who was 9 years old when enrolled in the trial) was milder than that seen in classic SCID (*19*). She had her first serious infection at age 3, and septic arthritis at age 5; the diagnosis was finally established at age 6 when significant lymphopenia with ADA deficiency was confirmed. This patient had an excellent initial improvement in peripheral T cell numbers after the start of PEG-ADA therapy (30 U/kg/week) at age 5, but lymphopenia recurred in the third and fourth years of enzyme treatment (Fig. 1B). During the year before gene therapy, repeated evaluation of her immune system showed persisting immunodeficiency, but less severe than that in patient 1. Despite 4 years of enzyme treatment, DTH skin test reactivity was absent (Table 1), cytotoxic T cells to viral antigens and allogeneic cells were deficient, and isohemagglutinins were barely detectable. However, illustrating the variability seen in the responses of patient 2 over time, blood lymphocytes that were cryopreserved from the day the clinical trial began and tested later showed normal cytotoxic activity to allogeneic cells.

Within 5 to 6 months of beginning gene therapy, the peripheral blood T cell counts for patient 1 (Fig. 1A) rapidly increased in number and stabilized in the normal range and have remained normal since that time (*20*). ADA enzyme activity, nearly undetectable in her blood lymphocytes initially, progressively increased in concentration during the first 2 years of treatment to reach a level roughly half the concentration found in heterozygous carriers (expressing only one intact ADA allele) and has re-

Table 1. DTH skin test reactivity and isohemagglutinin titers in sera of each patient at various times during the treatment protocol. Skin tests were applied as Multitest (Pasteur Merieux, Lyon, France) and scored according to the manufacturer's instructions 48 to 72 hours after being placed. Seven antigens were placed on the dates indicated, although only five were technically satisfactory on day 1252 for patient 1 and on day 1118 for patient 2. Isohemagglutinin titers were determined by standard blood bank techniques (*34*). Ninety five percent of normal children over the age of 2 years will have a titer of ≥1:16 and 82% will have a titer ≥1:32 (*35*). ND, not done. For the DTH skin tests, positive tests were elicited; T, tetanus toxoid; D, diphtheria toxoid; C, *Candida albicans*; P, Proteus antigen; S, streptococcal antigen; OT, old tuberculin.

Protocol day	Isohemagglutinins	DTH skin tests
	Patient 1	
−9	16	None (0/7)
115	256	ND
251	128	ND
314	32	T, D, C
455	32	T, D, C, S, P
510	64	ND
707	32	ND
1252	ND	D, C, P
	Patient 2	
−122	4	None (0/7)
−9	4	ND
90	256	ND
186	128	ND
291	128	ND
501	128	T, D, C, S, OT
676	64	ND
957	16	ND
1118	ND	T, D, S, P

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mained at that level since (Fig. 1A). Thus, both the reconstituted number of peripheral blood T cells and the elevated T cell ADA enzyme concentration have persisted since the patient's last treatment, indicating that peripheral T cells can have an unexpectedly long life-span and that gene expression from the retroviral vector has not been silenced over this period.

Patient 2, who had variable immune reactivity before enrollment, responded to the institution of lymphocyte infusions, with her peripheral T cell count rapidly increasing to levels in the high normal range (Fig 1B). Beginning with infusion 5, which included protocol modifications to partially deplete CD8 cells from the initially cul- -ed cell population (*21*), her T cell count into the mid-normal range, where it persisted throughout the treatment period and for a year after the last cell infusion. In contrast to those in patient 1, ADA enzyme levels in the circulating T cells of patient 2 did not rise significantly above the small amounts seen before gene therapy treatment (~1.5 nmol/10^8 cells per minute).

The differences in final lymphocyte ADA concentration are consistent with the levels of gene transfer reached in these patients. For several months in the second protocol year during which cell infusions were not given, LASN vector sequences detected by polymerase chain reaction (PCR) maintained a stable frequency in the peripheral blood of patient 1 at a level greater than the PCR-positive control standard containing the equivalent of 0.3 vector copies/cell (Fig. 2). By contrast, although vector-containing cells were also stably detected throughout a similar r -d in patient 2, their level reached only a e equivalent to 0.1 to 1.0% of her circulating cells carrying the inserted ADA vector.

The principal contributor to the difference in the final frequency of LASN vector–modified T cells in patients 1 and 2 was the low gene transfer efficiency in the cells of patient 2; this was consistently only a tenth or less of what was routinely achieved in the cells from patient 1. Despite the gross differences in the final proportion of vector-containing cells reached in these two patients, both CD4 and CD8 T cell populations from each have remained consistently positive for integrated vector sequences since the first infusion through protocol day 1480 for patient 1 and through protocol day 1198 for patient 2 (Fig. 2).

To more accurately measure the proportion of vector-containing cells in patient 1, we performed quantitative Southern (DNA) hybridization analysis for vector sequence on DNA isolated from her peripheral blood T cells at different days during the course of this protocol. On protocol days 816 and 1252, which represent samples taken 109 and 545 days after the last treatment, the vector concentration was at the level of approximately one vector copy per cell (Fig. 3). Longitudinal studies of samples obtained throughout the study show that this large amount of integrated vector was reached by infusion 8 (D707) and that it has remained in this range since that time (*22*).

The use of a restriction endonuclease that cuts only once within the vector sequence does not give detectable bands (Fig. 3), indicating that the population of blood T cells at these dates is not oligoclonal with respect to integrated vector. Vector-derived mRNA was readily detected by reverse transcription (RT)–PCR at these same times (Fig. 3), confirming that vector expression persisted and was correlated with the presence of ADA enzyme activity in her circulating T cells.

To evaluate the effect of gene therapy on the immune function of these two patients in addition to its beneficial effect on T cell numbers, we performed a panel of immunologic studies both before, and at various times after, treatment. DTH skin test reactivity to common environmental and vaccine antigens tests the overall competence of the cellular immune system because a response depends on the full complement of cellular functions, not just cell proliferation or secretion of a single cytokine (Table 1). Patient 1 was anergic before our protocol treatment despite nearly 2 years of PEG-ADA treatment. Eight months after the initiation of gene therapy (protocol day 251), she had a brisk DTH response to a single intradermal skin test with tetanus toxoid. By protocol day 455, DTH responses to five of seven antigens were present, and this increased responsiveness has persisted, through day 1252.

Before the protocol, patient 2 had no positive DTH skin test (Table 1). At protocol day 501, five positive DTH skin tests were elicited, and this increased DTH reactivity had persisted when she was last tested on day 1118. She also acquired palpable lymph nodes and visible tonsils during the period of protocol treatment.

To corroborate the improved immune function indicated by these DTH tests, we evaluated the capacity of peripheral T cells from our patients to produce interleukin-2 (IL-2) or to kill antigenic target cells in vitro. In several patients treated with PEG-ADA, in vitro T cell proliferative responses to mitogens may normalize, whereas responses to specific antigens are less improved (*7–10*). During PEG-ADA treatment before gene therapy, T cells from patient 1 produced IL-2 in response to stimulation with

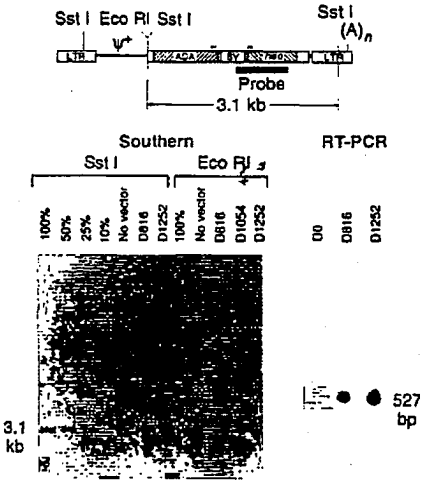

Fig. 3. Quantitative Southern hybridization analysis of DNA prepared from the blood mononuclear cells of patient 1 on protocol days (D) 816 and 1252 (*28*). DNA digested with Sst I should yield a single restriction fragment of 3.1 kb containing both the vector *neo* and ADA genes. Eco RI cuts only once within the vector sequence, and therefore a detectable band would indicate that a predominant clone with a single unique vector integration site was present in that blood sample. None was detected. Polyadenylated mRNA was extracted from the patient cells on days 0, 816, and 1252 and analyzed for vector message by RT-PCR (*29*). The primer locations used are indicated as short solid lines above the vector diagram. SV, SV40 early promoter; $(A)_n$, polyadenylation site; Ψ, extended retrovirus packaging signal. Hatched regions indicate protein coding regions.

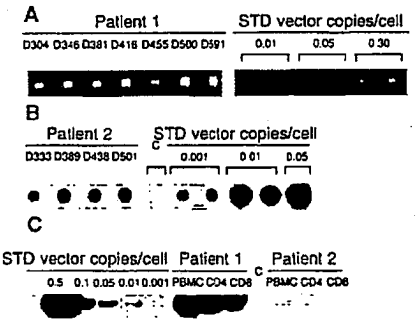

Fig. 2. PCR evaluation of the frequency of LASN vector–positive cells in the blood of patients 1 and 2 at various protocol days. (A) Cells from patient 1 for protocol days (D) 304 to 591 (see Fig. 1A). PCR analysis was performed as described (*26*) in an ethidium-stained gel. (B) Cells from patient 2 for protocol days (D) 333 to 501 (see Fig. 1B). PCR products were probed with ^{32}P-labeled *neo* gene as described (*26*). (C) Purified CD4$^+$ and CD8$^+$ cell subpopulations from patient 1 (D1480) and patient 2 (D1198) prepared by separation of peripheral blood mononuclear cells (PBMCs) by fluorescence-activated cell sorting (FACS). The purity of the separated T cell subpopulations from which DNA was extracted exceeded 98%, as confirmed by FACS analysis. Direct PCR with [^{32}P]deoxycytosine triphosphate was performed as described (*27*). Standards (STD) were prepared from DNA obtained from cell mixtures of a known proportion of LASN-transduced cells containing a single vector insert mixed with vector-negative cells. C, vector-negative control cells.

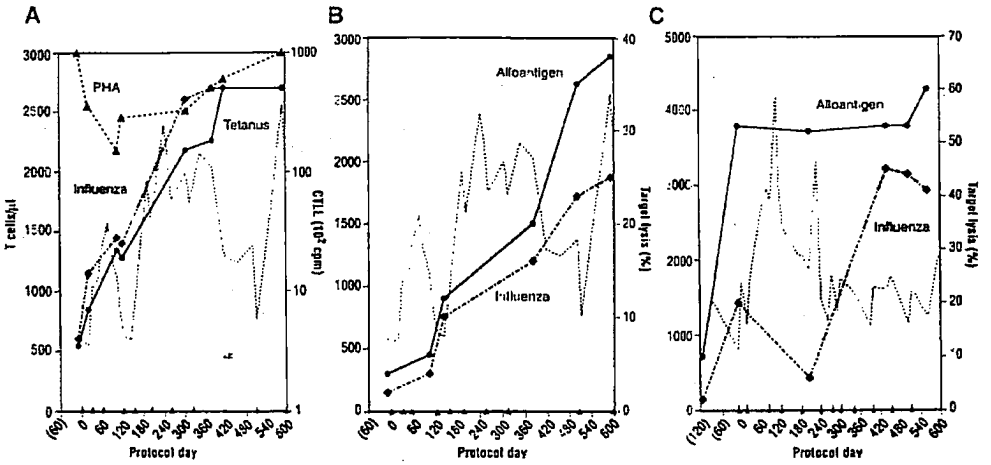

Fig. 4. Evaluation of the in vitro cellular immune responses of blood T cells from patients 1 and 2 at various times before and during the gene therapy trial. At least two normal subjects were included concurrently in each assay, and only those in which the controls responded appropriately are included here. (A) Production of IL-2 by cultured cells from patient 1 after stimulation with the mitogen PHA and with the specific antigens tetanus toxoid and influenza A virus as described (*30*). IL-2 was quantitated by bioassay measuring the proliferation of the IL-2–dependent T cell line CTLL at a 1:2 dilution of the lymphocyte culture supernatant. The fine dashed line indicates the patient's T cell count for reference. Solid triangles along the base line indicate the dates of cell infusion. (B) In vitro killing of a ^{51}Cr-labeled, influenza A–infected autologous B cell line and a ^{51}Cr-labeled allogeneic target B cell line by blood T cells from patient 1 as described (*31*). Lysis (as percent specific isotope release during a 6-hour incubation of effector and target cells at a ratio of 60:1) was measured after in vitro pre-stimulation for 7 days. Solid triangles along the base line indicate the dates of cell infusion. (C) In vitro killing of a ^{51}Cr-labeled, influenza A–infected autologous B cell and a ^{51}Cr labeled allogeneic target B cell line by blood T cells from patient 2 as described above.

the mitogen phytohemagglutinin (PHA) (Fig. 4A) but were unable to produce IL-2 in response to stimulation with influenza A virus or tetanus toxoid, despite repeated immunization with these antigens. Over the first months of gene therapy, IL-2 production improved and became normal after 1 year (Fig. 4A). Again before gene therapy, patient 1's T cells failed to show significant cytolytic reactivity against either allogeneic cells or influenza A–infected target cells. Almost mirroring the steady increase in IL-2 production, she acquired normal in vitro cytolytic T cell responses to these antigens, reaching normal values in her second year of treatment. (Fig. 4B).

The results of these cytolytic assays for patient 2 are shown in Fig. 4C. Tests done 120 days before the beginning of gene therapy also showed impaired responses. However, cells that were obtained at the time of the first gene therapy infusion, cryopreserved, and subsequently tested some months later showed a normal cytolytic response to allogeneic cells. After a year on gene therapy, cytolytic T cell activity against influenza also became normal.

To evaluate the effects of our treatment on humoral immune function in these patients, we measured antibody responses to several antigens. Despite their PEG-ADA treatment, both patients 1 and 2 had only low or borderline titers of isohemagglutinins on repeated testing before gene therapy. Each patient showed significant elevations in the levels of these antibodies within 90 to 115 days of beginning treatment with gene-modified cells (Table 1). Isohemagglutinins are antibodies that react with group A and B red blood cell antigens and occur spontaneously as a result of environmental exposure to cross-reacting antigens. Isohemagglutinin responses are, therefore, less dependent on the timing of previous immunizations than are responses to common vaccine antigens. After gene therapy, each patient also had improvement in antibody responses to vaccines to *Hemophilus influenzae* B (HIB) and tetanus toxoid (Fig. 5). With enzyme therapy alone, peripheral lymphocytes from each patient were unable to produce immunoglobulin M (IgM) in vitro after stimulation with pokeweed mitogen (PWM), but made robust responses after a year on the gene therapy protocol (Fig. 5A). Immunoglobulin production to PWM depends on T cells; these results further confirm the reconstitution of T cell function associated with gene therapy.

The effects of this treatment on the clinical well-being of these patients is more difficult to quantitate. Patient 1, who had been kept in relative isolation in her home for her first 4 years, was enrolled in public kindergarten after 1 year on the protocol and has missed no more school because of infectious disease than her classmates or siblings. She has grown normally in height and weight and is considered to be normal by her parents. Patient 2 was regularly attending public school while receiving PEG-ADA treatment alone and has continued to do well clinically. Chronic sinusitis and headaches, which had been a recurring problem for several years, cleared completely a few months after initiation of the protocol.

This trial of retroviral-mediated gene transfer shows that the survival of reinfused transduced peripheral blood T cells is prolonged in vivo; the erroneous assumption that T cells would not have such long-term survival was often cited as a potential problem with this treatment strategy. Patient 1 has had a normal total peripheral T cell count since the last cell infusion, and the proportion of her circulating T cells carrying vector DNA has remained stable over that period. Further, expression of the ADA transgene under the influence of the retroviral long terminal repeat (LTR) promoter has persisted for a long period in vivo without obvious extinction. There have been swings in the level of ADA enzyme in her peripheral lymphocytes throughout the period of observation, but the level of blood ADA enzyme activity at 4 years (protocol day 1480) is equivalent to that found immediately after the last cell infusion 2 years earlier (Fig. 1A). Although the data have not yet been completely analyzed, blood obtained after 5 years showed continuation of this trend with, again, a normal T lymphocyte count and an equivalent ADA level.

The mechanism by which our treatment aided immune reconstitution in patient 2 is less clear. The responses of patient 2 to some in vitro immunologic tests were variable before beginning our treatment protocol, ranging from little or no detectable response to nearly normal responses on the blood sample from the day gene therapy began. This patient produced a normal antibody response to immunization with bacteriophage φX174 about a year before beginning gene therapy (*8*). Although we have shown several examples of depressed cellular and humoral immune responses that strongly improved after gene therapy, this highly variable immune reactivity while patient 2 was on PEG-ADA therapy alone complicates interpretation of the contribution of our therapy. There was a temporal relation between initiation of gene therapy and a normalized peripheral T cell count,

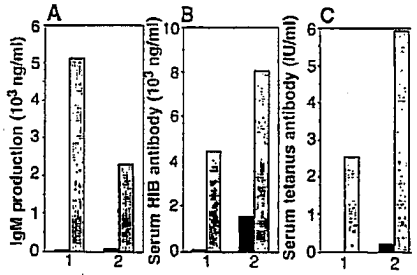

Fig. 5. Humoral immune function of patients 1 and 2 before (solid bars) and after (hatched bars) gene therapy. (A) IgM production by the patient's peripheral blood mononuclear cells in cultures stimulated with the T cell–dependent polyclonal activator PWM performed as described (*32*). "Before" samples were from D(−9). Follow-up cultures were at D500 (patient 1) and D560 (patient 2). In each case, the patient's cells stimulated with the T cell–independent B cell stimulant EBV (*33*) produced normal amounts of IgM (not shown), indicating intact B cell function before and after gene therapy, as expected. At least two normal subjects were included concurrently in each assay, and only those in which the controls responded appropriately are included here. (B) Serum antibody response to *Hemophilus influenzae* B. Patient 1 had failed to respond to two immunizations while on PEG-ADA alone [D(−9) shown]. Her response at protocol D591 is shown, after immunization. Patient 2 had some HIB-specific antibodies present before therapy [D(−122)], whose amounts increased without additional immunization during the protocol (D560). (C) Serum tetanus antibody. Patient 1 had negligible response to five separate tetanus immunizations before gene therapy [D(−48) shown] but responded briskly at D731, 24 days after re-immunization. Serum titers for patient 2 are shown for D(−9), 140 days after immunization while on PEG-ADA alone, and after receiving gene therapy (D592), 32 days after a booster tetanus immunization.

improved DTH, appearance of tonsils and palpable lymph nodes, normalized isohemagglutinin response, and improved PWM response, as well as other factors. In view of the relatively low level of ADA gene transfer achieved in this patient, the potential contribution of the infusions of the culture-activated T cells to the patient's response must also be considered. Perhaps ex vivo T cell activation somehow bypassed a differentiation block that PEG-ADA alone was unable to relieve. Despite the low final percentage gene transfer achieved, a 1% level of ADA gene–corrected cells could represent 10^9 to 10^{10} ADA-expressing T cells distributed throughout the body that could readily contribute to immune improvement.

Since the beginning of the trial, the dose of PEG-ADA enzyme given to each of our patients has been decreased by more than half (patient 1, 14 U/kg/week; patient 2, 10 U/kg/week), during which time their immune function has improved. By contrast, worsened immune function has been seen in other ADA$^-$ SCID patients when their dose of enzyme has been similarly reduced (*10*, *23*). We do not want to expose these patients to the potential risk of recurrent immunodeficiency by completely stopping PEG-ADA enzyme treatment until we have better information about the quality and duration of the immune improvement achieved by this first-generation gene therapy trial. The role of continued exogenous enzyme treatment will be clarified here or in companion studies attempting stem cell gene correction (*24*).

The safety of retroviral-mediated gene transfer has been a central concern. At least in the short and intermediate term, no problems have appeared in any clinical trial using these vectors. In the longer term, the theoretical potential for retroviral vectors to cause insertional mutagenesis remains the primary concern. To date, there has been no indication that malignancy associated with this process will be a complication of retroviral-mediated gene transfer.

Our trial here has demonstrated the potential efficacy of using gene-corrected autologous cells for treatment of children with ADA$^-$ SCID. Eleven children with this disease have been enrolled in various gene therapy protocols, each using different strategies and retroviral vector designs and focusing on different target cell populations. The experience gained from these approaches should provide guidance for gene therapy as a treatment for this disorder as well as for a larger array of inherited and acquired diseases.

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17. Peripheral T cells from the patients were collected by apheresis, isolated by density gradient centrifugation, washed extensively, and then cultured in 24-well culture plates in medium supplemented with 100 to 1000 IU/ml of recombinant IL-2 and 10 ng/ml of OKT3 to stimulate T cell proliferation. After 24 hours, half the medium was removed and replaced with supernatant containing the LASN retroviral vector supplemented with IL-2 and protamine (10 μg/ml) to give an initial multiplicity of infection of 1. The LASN vector contains the human ADA cDNA under the transcriptional control of the promoter-enhancer in the retroviral LTR and a neomycin phosphotransferase gene (*neo*) controlled by an internal SV40 promoter [R. A. Hock, A. D. Miller, W. R. A. Osborne, *Blood* 74, 876 (1989)] LASN was packaged with PA317 amphotropic retrovirus packaging cells (*2*). The LASN vector preparation, manufactured under good manufacturing practices by Genetic Therapy, Gaithersburg, MD, had a titer of 1×10^5 to 3×10^5. The cells were returned to the incubator and the transduction process repeated, with the addition of fresh retroviral supernatant and IL-2 twice daily for a total of three to five additions of vector. The cultured cells were transferred to gas-permeable culture bags at the conclusion of the transduction process. The proliferating T cell cultures were observed daily, split, and fed as necessary with periodic samples tested for viability and microbial contamination. Gene transfer efficiency was variable from treatment to treatment and patient to patient, ranging from 1 to 10% for patient 1 and 0.1 to 1% for patient 2. On days 9 to 12, the cultured cells were washed extensively with saline containing 0.5% human albumin and were then infused into the patient over a period of about 1 hour. During the 9 to 12 days of culture, the cell populations had expanded 17- to 135-fold. Preliminary studies testing the T cell receptor β gene repertoire showed that T cell cultures remained polyclonal for at least 3 weeks under these culture conditions. The culture period used in the clinical trial was held to half this time period to ensure a polyclonal T cell repertoire in the infused cell population.
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20. The protocol was reviewed and approved by the Clinical Research Subpanels of the NCI and NHLBI, the NCI Cancer Treatment and Evaluation Program (CTEP), the NIH Biosafety Committee, the Human Gene Therapy Subcommittee, the Recombinant DNA Advisory Committee, the Director of NIH, and the U.S. Food and Drug Administration. Informed consent was obtained from the parents of each patient.
21. Beginning with culture 9 for patient 1 and culture 5 for patient 2, the patients' lymphocyte populations obtained by apheresis were fractionated by adherence to flasks coated with CD8 monoclonal antibodies (Applied Immune Sciences) following the manufacturer's instructions. This protocol modification for CD8 depletion was introduced because both patients were developing a progressively inverted CD4-CD8 ratio. This effect was apparently the result of preferential growth of $CD8^+$ cells during the last 4 to 5 days of culture and the subsequent persistence of these infused $CD8^+$ cells in the circulation. Consequently, each subsequent apheresis sampled the recently increased number of $CD8^+$ cells, and thus the skewing of the ratio of CD4 to CD8 cells became compounded with each additional treatment. By partially depleting the apheresis sample of $CD8^+$ cells by an immunoaffinity selection process, the later treatments for each patient consisted of cells with a more balanced phenotype. The perturbation in normal CD4-CD8 cell proportions did not have detectable untoward effects for either patient.
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29. RT-PCR analysis for LASN vector transcripts was as follows: 3 μg of polyadenylated RNA was treated with deoxyribonuclease and reverse-transcribed. The cDNA (0.3 μg) was amplified with LASN vector–specific primers in a 30-cycle PCR reaction. The oligonucleotides 5′-CAGCCTCTGCAGGGCAGAAC-3′ (corresponding to the 3′ end of the ADA gene in LASN) and 5′-GCCCAGTCATAGCCGAATAG-3′ (complementary to 5′ end of the *neo* gene in LASN) were used as primers. After electrophoresis and blotting, the sequences were hybridized with a 527-bp probe corresponding to the entire length of the predicted PCR product.
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Physical Map and Organization of *Arabidopsis thaliana* Chromosome 4

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A physical map of *Arabidopsis thaliana* chromosome 4 was constructed in yeast artificial chromosome clones and used to analyze the organization of the chromosome. Mapping of the nucleolar organizing region and the centromere integrated the physical and cytogenetic maps. Detailed comparison of physical with genetic distances showed that the frequency of recombination varied substantially, with relative hot and cold spots occurring along the whole chromosome. Eight repeated DNA sequence families were found in a complex arrangement across the centromeric region and nowhere else on the chromosome.

Arabidopsis thaliana has been adopted as a model organism for the analysis of complex plant processes by means of molecular genetic techniques (*1*). The increase in map-based cloning experiments makes the generation of a complete physical map of the *Arabidopsis* genome a high priority. In addition, the availability of such a map would enable the organization of the chromosome to be studied in more detail. Little is known about the organization of plant chromosomes, but the general picture is that of chromosomes carrying large numbers of dispersed [often retrotransposons (*2*)] and tandemly repeated DNA sequences (*3*). The relatively small (100 Mb) *Arabidopsis* genome has a much smaller number of repeated DNA sequences than do most other plant species; its five chromosomes contain ~10% highly repetitive and ~10% moderately repetitive DNA (*4*). The dispersion of most of these sequences among the low-copy DNA is unknown.

We discuss here a physical map, which we have presented on the World Wide Web (WWW) at URL: http://nasc.nott.ac.uk/JIC-contigs/JIC-contigs.html, of *Arabidopsis* chromosome 4, one of the two chromosomes carrying nucleolus organizing regions. The construction of this map allowed us to analyze the frequency of recombination along the whole chromosome, the integration of the physical with the cytogenetic map, the interspersion pattern of repeated and low-copy DNA sequences over the whole chromosome, and the arrangement of repeated DNA sequences over the centromeric region.

We generated the physical map by hybridizing probes to four yeast artificial chromosome (YAC) libraries (*5*), using colony hybridization experiments (*6*). The probes consisted of 112 markers genetically mapped to chromosome 4, 20 previously unmapped genes, random genomic fragments and sequences flanking transposable elements, and the 180–base pair (bp) repetitive element carried in pAL1 (*7*). Southern (DNA) blot analysis of YAC clones confirmed the colony hybridization results and revealed common restriction fragments in the different YAC clones hybridizing to a given marker. This demonstrated overlap between the inserts of the YAC clones. On the basis of these results, the YAC clones could be placed into 14 YAC contigs with a high degree of redundant YAC cover, ensuring an accurate map despite the presence of chimeric clones in the YAC libraries.

We generated YAC end fragments, using either inverse polymerase chain reaction (IPCR) or plasmid rescue (*8*), from YAC clones lying near the ends of each of the 14 contigs. The fragments were hybridized to Southern blots of YAC clones from adjacent contigs. In addition, YACs, as well as some of the end fragments generated by IPCR, were used to identify clones from a cosmid library of the Columbia ecotype (*9*). The cosmids were then used as new markers on the YAC libraries. These experiments reduced the number of contigs to four. In all but two instances, the end fragments revealed that the contigs were already overlapping. Experiments aimed at closing the last three gaps have been attempted

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Exhibit C

article

Successful *ex vivo* gene therapy directed to liver in a patient with familial hypercholesterolaemia

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An *ex vivo* approach to gene therapy for familial hypercholesterolaemia (FH) has been developed in which the recipient is transplanted with autologous hepatocytes that are genetically corrected with recombinant retroviruses carrying the LDL receptor. We describe the treatment of a 29 year old woman with homozygous FH by *ex vivo* gene therapy directed to liver. She tolerated the procedures well and *in situ* hybridization of liver tissue four months after therapy revealed evidence for engraftment of transgene expressing cells. The patient's LDL/HDL ratio declined from 10–13 before gene therapy to 5–8 following gene therapy, improvements which have remained stable for the duration of the treatment (18 months). This represents the first report of human gene therapy in which stable correction of a therapeutic endpoint has been achieved.

Familial hypercholesterolaemia (FH) has emerged as an important model for the development of human gene therapies[1]. This disorder, caused by inherited deficiency of LDL receptors, is associated with severe hypercholesterolaemia and premature coronary artery disease[1]. The homozygous form of FH is an excellent candidate for early applications of gene therapy because it is a lethal disorder that is refractory to conventional therapies. Measurement of serum lipid profiles provides a convenient and clinically relevant endpoint to evaluate response to therapy, and orthotopic liver transplantation has been shown to correct the underlying dyslipidemia indicating that hepatic reconstitution of LDL receptor expression is sufficient for metabolic correction[2,3].

The original paradigm for liver-directed gene therapy was based on transplantation of autologous hepatocytes genetically modified *ex vivo* with recombinant retroviruses. The efficacy and safety of this approach for treatment of FH has been demonstrated in a variety of animal models. A strain of rabbits genetically deficient in LDL receptors, called the Watanabe Heritable Hyperlipidemic (WHHL) rabbit, was used to demonstrate the potential efficacy of *ex vivo* gene therapy. Analysis of recipient animals demonstrated stable engraftment of genetically modified hepatocytes and persistent reductions in serum cholesterol for the duration of the experiment — 6.5 months[4]. Experiments in larger animals including dogs and baboons documented the feasibility and safety of *ex vivo* gene therapy directed to the liver[5,6]. *In situ* hybridization analysis of liver tissue from baboons harvested 1.5 years after gene therapy demonstrated stable engraftment of transgene expressing hepatocytes, providing further support for the efficacy of this therapy (unpublished data).

Based on the encouraging results obtained in animal models, we proposed a clinical protocol to treat FH homozygous patients with *ex vivo* gene therapy. We received permission from the US Recombinant DNA Advisory Committee (RAC) and the US Food and Drug Administration to treat three patients who had developed overt coronary artery disease and therefore would have a poor prognosis. Our experience with the first patient, described here, supports the efficacy and safety of liver-directed *ex vivo* gene therapy in humans.

First recipient of liver-directed gene therapy

The first clinical application of liver-directed gene therapy in humans used the *ex vivo* approach in a patient with homozygous FH. Although we were allowed to treat FH patients of any age, the RAC suggested that we enroll an adult as the initial patient to simplify the informed consent process. Patient FH1 underwent gene therapy on June 5, 1992. This French Canadian woman, who at the time of gene therapy was 28 years old, had a myocardial infarction at the age of 16 and required coronary artery bypass at the age of 26. Her dyslipidemia — which at baseline included a total serum cholesterol concentration of 545 mg dl^{-1}, LDL of 482 mg dl^{-1} and HDL of 43 mg dl^{-1} — was refractory to treatment with a variety of drugs including HMG CoA reductase inhibitors and bile acid binding resins. Genotype analysis indicated she was homozygous for a missense mutation (Trp66Gly, exon 3) that renders the LDL receptor incapable of binding to its ligands[7]. Cardiac evaluation performed prior to gene therapy revealed failure of one of her grafts and diffuse disease in her native coronary arteries, however, she was not overtly symptomatic.

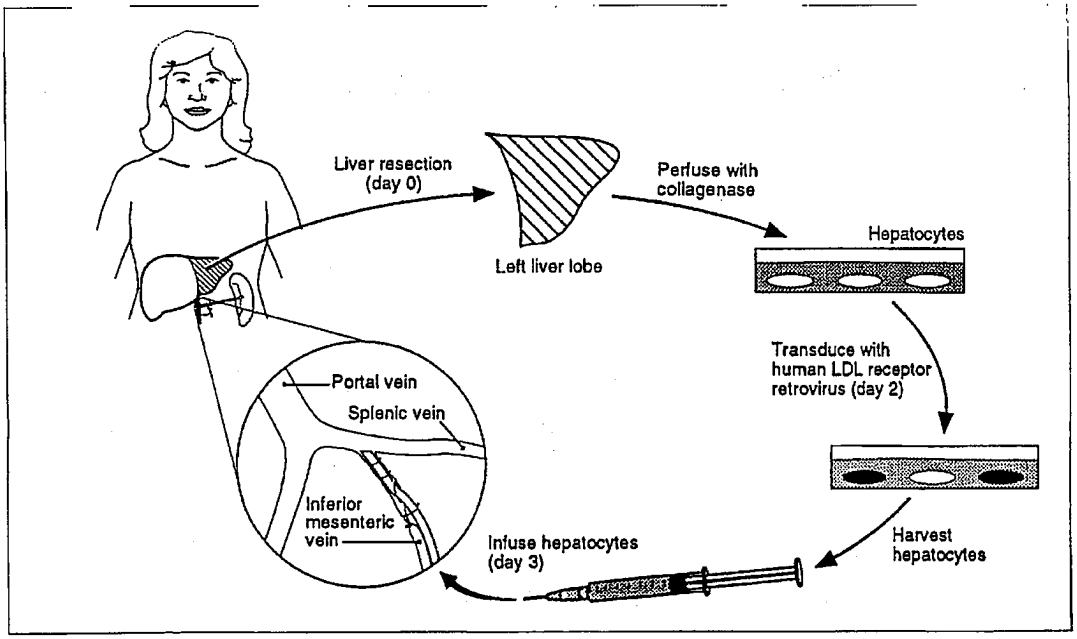

Fig. 1 Strategy of *ex vivo* gene therapy for familial hypercholesterolaemia.

Ex vivo gene therapy to liver is feasible and safe

The clinical protocol approved by the RAC has been published[8]; the general strategy is summarized in Fig. 1. The left lateral segment of the patient's liver, comprising approximately 15% of its total mass, was removed through a left subcostal incision. A 9.6 fr Hickman catheter was inserted into her inferior mesenteric vein, and the distal end of the catheter was brought through her incision thereby providing convenient access to the portal circulation for subsequent cell infusions. The resected liver, weighing 250 g, was perfused with collagenase to release hepatocytes; 3.2×10^9 cells were recovered (98% viability) and seeded into 800 10 cm^2 plates. Medium containing the LDL receptor expressing recombinant retroviruses was placed onto the cultured hepatocytes 48 hours after the initial seeding. Following a 12–18 h exposure to virus, the cells were analysed for LDL receptor expression and harvested for transplantation; 2×10^9 viable cells were recovered from the plates by treatment with trypsin. Incubation of the transduced cells with fluorescent labelled LDL revealed uptake in approximately 20% of the cells exposed to the LDL receptor expressing virus and no uptake in duplicate plates of cells not exposed to virus (Fig. 2).

Prior to infusion of the cells, a portal venogram was performed to confirm the placement of the catheter and patency of the portal circulation (Fig. 3*a*). The genetically corrected hepatocytes were harvested in three aliquots and each aliquot was manually infused at 4 h intervals directly into the catheter over a 30 minute period (a rate of ~2 cc min^{-1}). During the cell infusions the patient was carefully monitored in the intensive care unit; her vital signs measured during this time are presented in Fig. 4. She tolerated the cell infusions well except for a transient tachycardia early in the day, thought to be secondary to anxiety, and fevers that were present before cell infusion which resolved subsequently.

One concern was the potential development of portal

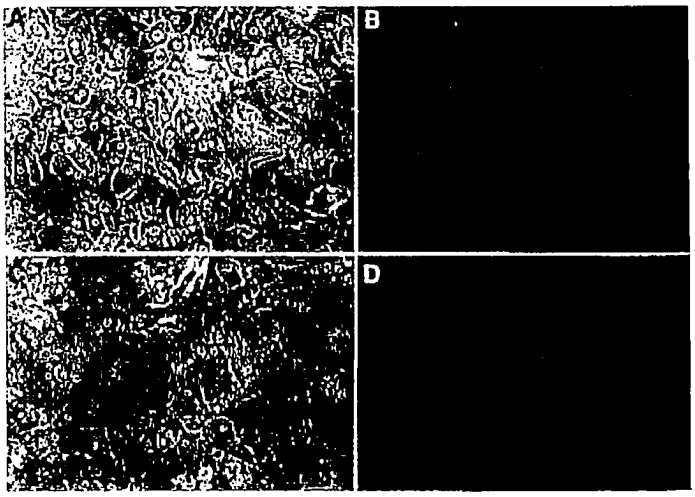

Fig. 2 Expression of recombinant LDL receptor in primary hepatocytes. Primary cultures of hepatocytes were infected with recombinant retroviruses and analysed for LDL receptor expression using an assay based on uptake of fluorescent labelled LDL. Mock infected cells visualized under the phase contrast (*a*) and fluorescent microscope (*b*), and LDL receptor transduced cells visualized under the phase contrast (*c*) and fluorescent microscope (*d*) are presented. Magnification, 100×.

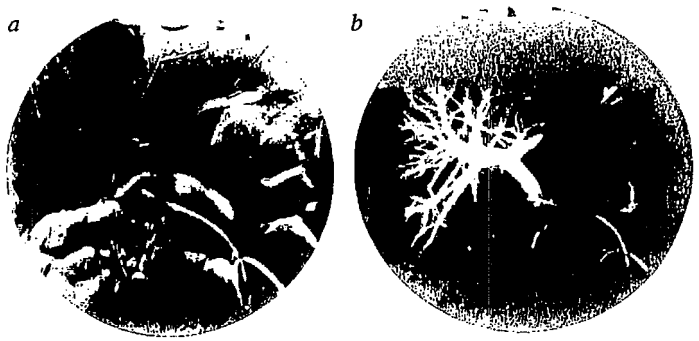

Fig. 3 Portal venograms before and after hepatocyte infusion. *a*, Portal venogram immediately prior to cell reinfusion (postoperative day 3). Note surgical absence of the left lateral segment of the liver, absence of portal vein thrombosis and good position of the catheter. *b*, Portal venogram immediately prior to catheter removal (postoperative day 10). Note patent portal vein without evidence of intraluminal thrombus.

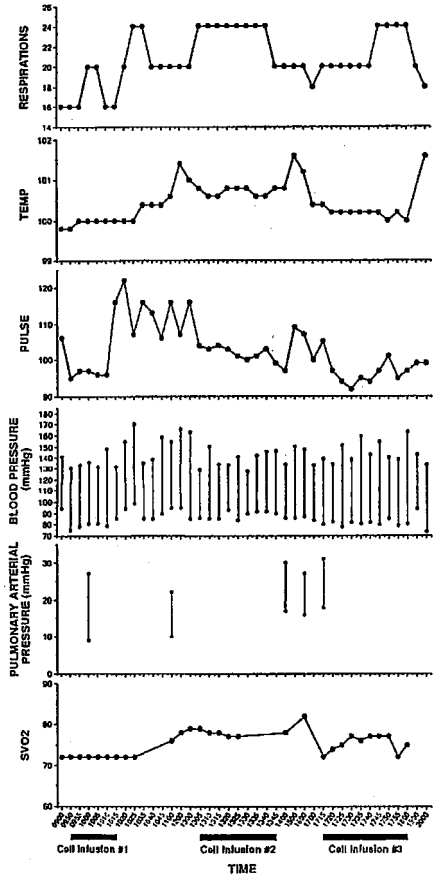

Fig. 4 Clinical response during hepatocyte infusion. During the cell infusions the patient was invasively monitored with a radial arterial line and pulmonary arterial line while in the intensive care unit. Six clinical parameters (respiration rate, oral temperature, pulse, systemic blood pressure (mm Hg), pulmonary arterial pressure (mm Hg), and oxygen saturation in mixed venous blood (SV02)) are presented. The actual times are indicated along the bottom with the periods during which the cells were infused indicated by the bars. Note that the time coordinates have been expanded during the periods of cell infusion.

vein thrombosis and/or portal hypertension as a result of introducing a large cell mass into this low pressure venous circulation. Portal pressures measured via the catheter three days before cell infusion (9.8 ± 1.3, mean ± s.d., n=4) were indistinguishable from those measured five days after cell infusion (10.9 ± 2.1 mean ± s.d. n=12) with transient increases (lasting <4 h) of 4 and 8 cm H_20 occurring after the second and third cell infusions, respectively. Repeat portal venography performed at the time of catheter removal seven days after cell infusion revealed a fully patent portal circulation without evidence of intraluminal clot (Fig. 3*b*).

Prolonged improvement in dyslipidemia

Liver tissue was harvested by percutaneous biopsy four months after gene therapy. No histopathology was noted in plastic embedded sections prepared for light and electron microscopy (data not shown). Frozen sections were analysed for the presence of transgene expressing cells by *in situ* hybridization using an RNA probe specific for the recombinant derived LDL receptor transcript. Figure 5 presents an example of an hepatocyte that hybridized to the antisense probe (*c* and *d*); this kind of focal hybridization was not present in serial sections incubated with the sense probe (*a* and *b*) or in sections pretreated with RNase prior to hybridization with the antisense probe (data not shown). Analysis of a limited number of sections revealed hybridization to the antisense probe in 1:1,000 to 1:10,000 cells. It is unlikely that the results obtained from a single small block of liver tissue from FH1 accurately represents the abundance and distribution of transgene expressing cells throughout the liver. Similar experiments performed in three baboons who underwent

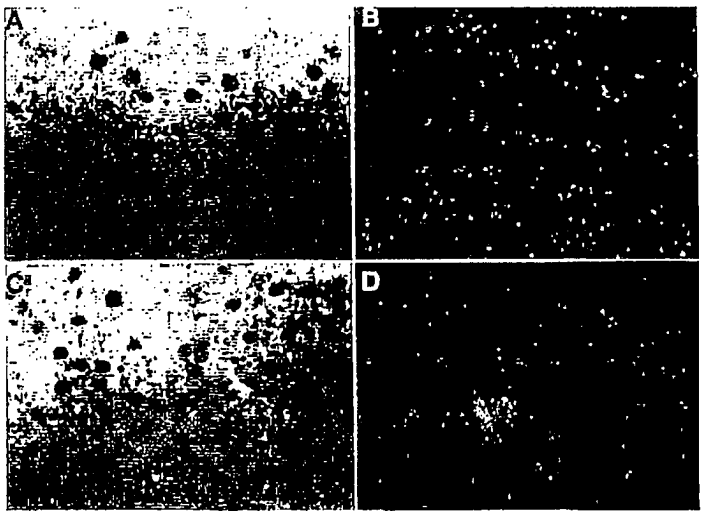

Fig. 5 *In situ* hybridization of liver tissue after gene therapy. Liver tissue (50 mg) was harvested by percutaneous biopsy four months after gene therapy. The majority of the sample was fixed, embedded, sectioned and analysed by light and electron microscopy for evidence of pathology. A small block was analysed for cells expressing recombinant LDL receptor by *in situ* hybridization. Tissue sections were hybridized with the sense probe (*a* and *b*) or antisense probe (*c* and *d*) and visualized by bright field (left panels) and dark field (right panels) microscopy. The clustering of signal seen in panels *c* and *d* indicates a cell that hybridized to the antisense probe. Magnification, 50×.

ex vivo liver-directed gene therapy demonstrated marked regional variation of recombinant LDL receptor expressing cells within large biopsies of liver, illustrating the limitations in quantitatively assessing engraftment from the small quantity of tissue sampled from a percutaneous biopsy (unpublished data).

The effect of gene therapy on the patient's lipid profiles, presented as ΔLDL, ΔHDL and LDL/HDL ratio, is presented in Fig. 6; Table 1 summarizes the lipid data with relevant statistical analyses. The patient has been followed for 18 months after gene therapy in the context of four treatment periods: pre gene therapy — on (period A) and off (period B) lovastatin; and post gene therapy — off (period C) and on (period D) lovastatin. The original protocol was designed to establish baseline lipids when the patient was off all lipid lowering medications and to reinitiate pharmacologic therapy four months after gene therapy. This initial analysis allowed comparisons between periods B, C and D.

Blood samples were coded and submitted to a reference

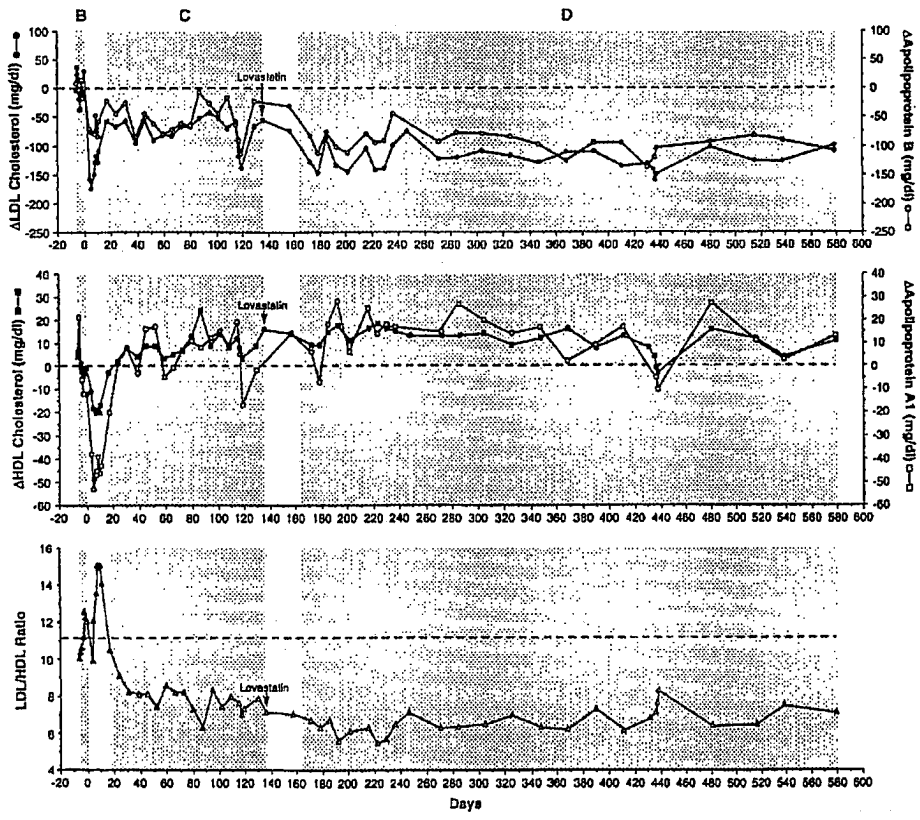

Fig. 6 Lipid profiles. The study was performed using three treatment periods. Period B spans 8 days immediately prior to gene therapy during which 7 lipid profiles were obtained. Period C represents a 131 day interval after gene therapy before she was started on lovastatin during which 19 lipid profiles were obtained. Interpretation of data obtained 8 days following gene therapy was confounded because of additional effects on lipids of the stress of the procedure and decreased nutritional intake; these data were deleted from the analysis of Period C. Period D represents a 15 month interval following Period C during which the patient was treated with lovastatin. Data obtained during a 30 day period after initiation of lovastatin was not included in analysis of Period D to allow for the effect of the drug. Data are presented as ΔLDL and Δapo B (top panel); ΔHDL and Δapo AI (middle panel); and LDL/HDL ratio (bottom panel). Three treatment periods are indicated: period B — pre-gene therapy, off medications; period C — post-gene therapy, off medications; and period D — post-gene therapy, on lovastatin.

Table 1 Summary of lipid profiles for patient FH1

	Pre-gene therapy		Post-gene therapy		Statistical comparisons (p values)		
	+Lovastatin period A	−Lovastatin period B	−Lovastatin period C	+Lovastatin period D	B–C	C–D	A–D
Cincinnati ref. lab.							
LDL	–	482 ± 19 (7)	404 ± 24 (19)	356 ± 22 (27)	0.0001	0.0001	–
HDL	–	43 ± 3.4 (7)	51.4 ± 5.9 (19)	54 ± 5 (27)	0.0014	0.10	–
LDL/HDL	–	11 ± 1.0 (7)	7.9 ± 0.9 (19)	6.6 ± 0.6 (27)	0.0001	0.0001	–
apoAI	–	115 ± 12 (7)	121 ± 11 (18)	130 ± 9 (24)	0.22	0.005	–
apoB	–	352 ± 23 (7)	299 ± 30 (18)	260 ± 23 (24)	0.0004	0.0002	–
Quebec ref. lab.							
LDL	448 ± 30 (6)	–	–	366 ± 25 (7)	–	–	0.0001
HDL	44.5 ± 4.1 (6)	–	–	47.6 ± 4.1 (7)	–	–	0.16
LDL/HDL	10.2 ± 1.0 (6)	–	–	7.6 ± 0.6 (7)	–	–	0.0001

All samples represent mean ± 1. s.d. with *n*=number of determinations. Statistical analyses performed as described in the text.

laboratory in Cincinnati for analysis. Serum LDL dropped by 180 mg dl^{-1} immediately after cell infusion and regained a new baseline that was 17% lower than pre treatment levels (482±19 before therapy versus 404 ± 24 after therapy, p=0.0001). Coincident with the diminution in LDL was an increase in HDL from 43 ± 3.4 to 51.4 ± 5.9 (p=0.0014) that translated to a decline in LDL/HDL ratio from 11 ± 0.4 to 7.9 ± 0.9 (p=0.0001). The mechanism(s) responsible for increased HDL following gene therapy remain unexplained, however, similar effects have been described in FH homozygotes who underwent orthotopic liver transplantation[2,3]. Initiation of lovastatin four months after gene therapy was associated with further improvements in this patient's dyslipidemia including a reduction in LDL (404 ± 24 to 356 ± 22, p=0.0001), increase in HDL (51.4 ± 5.9 to 54 ± 5, p=0.10), and decline in LDL/HDL ratio (7.9 ± 0.9 to 6.6 ± 0.6, p=0.0001). The changes in LDL and HDL noted in each treatment period were associated with parallel and equally significant changes in apo B and apo AI, respectively (Table 1 and Fig. 6).

An attempt was made to evaluate further the effect of gene therapy using lipid profiles that were obtained in Quebec during period A, spanning a two year interval from Feb. 1990–Dec. 1991; during this time the patient was treated with lovastatin in a single drug regimen at approximately the same dose she has been taking during period D (that is, following gene therapy). Additional samples were obtained during period D and analysed by the same reference laboratory in Quebec that was used to measure the patient's lipids in 1990–91. Direct comparison of the patient's lipids on lovastatin before and after gene therapy revealed a diminution in LDL from 448 ± 30 to 366 ± 25 (p=0.0001), a modest increase in HDL from 44.5 ± 4.1 to 47.6 ± 4.1 (p=0.16), and a decline in LDL/HDL ratio from 10.2 ± 1.0 to 7.6 ± 0.6 (p=0.0001).

Discussion

FH represents a unique model for developing and evaluating the principle of liver-directed gene therapy in humans. This fatal disease is easily evaluated for reconstitution of hepatic gene expression by serial measurements of serum lipids which are considered relevant endpoints for clinical efficacy. Critical to the early development of this clinical model was the availability of an authentic animal model, the WHHL rabbit.

Ex vivo approaches to liver-directed gene therapy emerged as the initial paradigm for treating hepatic metabolic diseases such as FH. In this strategy, stable reconstitution of hepatic gene expression can be achieved by transplanting hepatocytes transduced *ex vivo* with retroviruses. The development of safe and effective *ex vivo* gene therapies to liver presents unique experimental challenges. *Ex vivo* correction of the defect is complicated because the target cell for gene transfer, the hepatocyte, must be isolated from surgically resected tissue and it cannot be maintained and expanded in culture. The ultimate success of this approach depends on the efficient and stable engraftment of the transduced cells and their progeny. The likelihood that this will occur with transduced hepatocytes is difficult to predict because of the paucity of information available regarding stem cells and lineage in the liver, and ultimately must be answered experimentally. Clinical application of this form of gene therapy was further confounded because it does not resemble existing forms of therapy as is the case with bone marrow directed gene therapy, which conceptually is a modification of a commonly used therapy, autologous bone marrow transplantation. However, there should be no immunological barriers associated with *ex vivo* gene therapy other than the problem of an immune response to the therapeutic gene product, a potential concern that is generic to all forms of gene therapy for deficiency states. A variety of animal models, in addition to the WHHL rabbit, have been useful in developing the requisite technology and providing sufficient preclinical studies to justify a human trial[4,9,10].

The outcome of our first clinical experience supports the safety and feasibility of *ex vivo* gene therapy directed to liver. Molecular and metabolic data suggest that the genetically modified hepatocytes have engrafted stably in this patient and continue to express the recombinant gene (after at least 18 months). The level of metabolic correction achieved in this patient was similar to that detected in the WHHL rabbits who received autologous genetically corrected hepatocytes[4]. In this animal model, control experiments performed with mock transfected hepatocytes had no effect on cholesterol except for a transient elevation suggesting that the persistent diminution in lipoproteins observed in FH1 was not an artefact of the surgical

procedures but due to expression of the recombinant gene. Subsequent to gene therapy, the patient's serum lipids consistently remained at levels significantly lower than those measured by at least two reference laboratories over several years before gene therapy. It is unclear, however, whether the partial correction of hypercholesterolaemia achieved in this patient will translate to improved clinical outcome. It is encouraging that she tolerated gene therapy well without short or long term sequelae and that her coronary artery disease, as documented by serial angiography, has not progressed during the 18 months since the treatment (data not shown).

The response of this patient to lovastatin following gene therapy is interesting given that she failed to respond to this drug on multiple occasions prior to gene therapy. Lovastatin is thought to deplete intracellular cholesterol which leads to up regulation of LDL receptor expression[1], probably at the transcriptional level[11]. The recombinant LDL receptor gene does not contain the transcriptional elements necessary to confer cholesterol mediated regulation suggesting the response to lovastatin is unrelated to the recombinant gene or that its effect is in part mediated by posttranscriptional regulation of LDL receptor. This is consistent with previous studies that indicate the endogenous LDL receptor gene is regulated at both a transcriptional and posttranscriptional manner[12].

One potential concern about gene therapy for diseases caused by loss of gene function is that the protein product of the therapeutic gene will be recognized by the recipient as a neoantigen leading to an immune response against the genetically corrected cells. Several observations suggest this did not occur in FH1. Western blot analysis of the patient's sera failed to detect antibodies to the recombinant human LDL receptor protein (data not shown). Also, there was no clinical or pathological evidence for autoimmune hepatitis following gene therapy. It will be interesting to see if similar results are obtained in FH patients undergoing gene therapy who have mutations that totally ablate LDL receptor protein expression as opposed to the mutation in FH1 that leads to the expression of a dysfunctional protein[7].

Our study demonstrates the feasibility, safety and potential efficacy of *ex vivo* liver-directed gene therapy in humans and supports the initial hypothesis that selective reconstitution of LDL receptor expression in hepatocytes of FH homozygotes should be sufficient for metabolic improvement. This represents the first example of stable correction of a therapeutic endpoint by gene therapy, in contrast to clinical trials that require repeated administration of short-lived target cells such as lymphocytes for treatment of adenosine deaminase deficiency. Translation of this technology to the treatment of other lethal liver metabolic diseases (such as, ornithine transcarbamylase deficiency) should proceed rapidly if the principle of *ex vivo* liver-directed gene therapy is confirmed in a larger number of homozygous FH patients. Ultimately, a more effective and clinically practical approach to liver directed gene therapy, based on *in vivo* gene delivery, must be developed. Gene transfer technologies using recombinant adenoviruses, liposomes and molecular conjugates have shown promising results in animal models[13–15]. Problems with efficiency and stability of recombinant gene expression as well as destructive and/or blocking immune responses to the delivery vehicles or genetically modified cells must be overcome before the potential of *in vivo* approaches can be realized.

Methodology

Surgical procedures. During the procedure and for the first three postoperative days the patient was carefully monitored with a pulmonary arterial catheter and radial arterial line. Following induction of anesthaesia, the left lobe of the liver was exposed by a left subcostal incision which was extended up the midline to the xiphoid. A self-retaining retractor was used to retract the costal margin. The left triangular ligament was divided to the level of the left hepatic vein from lateral to medial. A rubber-shod, non-crushing intestinal bowel clamp was tested for fit just to the left of the falciform ligament. The clamp was applied and using a scalpel, the liver surface was rapidly transected (<90 s) and transferred to the human applications laboratory for cell isolation. Bleeding from the cut hepatic vein was easily controlled with direct pressure until surgical hemostasis was applied. The cut ends of the portal vein and hepatic vein were sutured with 5-0 running non-absorbable monofilament. The open surface of the liver was controlled with 3-0 silk interlocking vertical mattress sutures placed in the liver tissue protruding from the clamp. Once haemostasis was achieved, the clamp was removed and individual bile ducts or blood vessels were ligated with additional 3-0 silk sutures. The inferior mesenteric vein was identified at the paraduodenal fossa. The vein was sharply dissected for a distance of 3 cm and individual branches were ligated with 5-0 silk ligatures. Silk ties (2-0) were placed at either end of the dissected vessel, and the ligature placed at the end of the vein nearest to the colon was tied. A 9.6 Fr. Hickman-type catheter was brought obliquely through the abdominal wall about 3 cm below the lateral aspect of the incision and secured in place with a 3-0 nylon stitch. The catheter was trimmed to the correct length, a bevel was placed at the cut end to facilitate insertion, and the catheter was primed with heparinized saline (100 U ml^{-1}). A venotomy was made with a number 11 scalpel blade. The ideal location for catheter placement is the confluence of the inferior mesenteric vein and the splenic vein, a position that was identified by palpation. The catheter was secured by tying the ligature nearest the portal vein around the inferior mesenteric vein making sure not to occlude the catheter. A 3-0 chromic suture was tied around the inferior mesenteric vein and the outside of the catheter to further protect against premature dislodgement (see Fig. 1). A final inspection of the cut surface of the liver was made and the liver bed was drained with a closed suction drainage catheter to remove any residual bile or serum. The wound was closed in two fascial layers with a running absorbable monofilament suture. The skin was closed with interrupted subcuticular 4-0 chromic sutures and surgical tapes.

Preparation of virus and isolation of hepatocytes. The recombinant retrovirus used in this study has been described[9]. A full length human LDL receptor cDNA is expressed from a chicken β-actin promoter in combination with an enhancer from the immediate early gene of cytomegalovirus. The cell line that produced this virus, called 132-10, was characterized in accordance with recommendations of the RAC and the FDA. Supernatants containing the LDL receptor viruses were cryopreserved and aliquots were analysed for the presence of contaminants and replication competent virus. Certified lots of cryopreserved virus were used in the clinical trial.

Hepatocytes were isolated by collagenase perfusion, plated in culture and infected with retroviruses as described previously[16]. Plates of cells were infected with virus from 132-10 (LDL receptor virus) and analysed for LDL receptor expression using the previously described assay; cells were incubated in RPMI 1640 medium containing lipoprotein deficient serum (10%) and fluorescent labelled LDL (10 μg ml^{-1}) for 4 h[16]. Following completion of this incubation, the medium was removed, and the cells were washed in PBS and visualized under the fluorescent microscope. In preparation for transplantation, hepatocytes were removed from the plates by incubation with trypsin and washed extensively in RPMI 1640. Hepatocytes were harvested in three batches each of which contained cells recovered from one third of the total prep. Each batch was washed and resuspended in normal saline (50 ml) containing 10 U ml^{-1} of heparin in preparation for infusion.

Analyses of biopsied liver tissue. The tissue block for *in situ* analysis

was frozen in OCT, and cryosections (6 μM) were mounted on gelatin poly(L-lysine)-coated sides and fixed with 4% paraformaldehyde in phosphate buffered saline[17]. Sections were hybridized to a ^{35}S labelled RNA probe complementary to retroviral envelope sequences that are uniquely present in the 3' untranslated region of the recombinant derived LDL receptor RNA[4]. Sense probes and RNAse pretreatment with antisense probes were used as controls for hybridization specificity.

Analysis of metabolic parameters. Blood samples were obtained, coded and sent to reference laboratories in Cincinnati and Quebec for determination of lipid profiles. LDL cholesterol, HDL cholesterol, ApoA1 and ApoB were measured directly using previously published techniques. Differences in LDL, HDL and LDL/HDL profiles obtained during the four treatment periods were evaluated using random intervention testing and randomized testing methodologies.

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